PB83-113290

Compilation of Ames 'Salmonella typhimurium' Plate Incorporation Test Protocols

(U.S.) Health Effects Research Lab.
Research Triangle Park, NC

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EPA-600/2-82-007
June 1982

PB83-113290

COMPILATION OF AMES *Salmonella typhimurium* PLATE INCORPORATION TEST PROTOCOLS

by

Stephanie Toney
Northrop Services, Inc. - Environmental Sciences
Research Triangle Park, NC 27709

and

Larry D. Claxton
Genetic Toxicology Division
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711

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Contract No. 68-02-2566

Project Officer

Larry D. Claxton
Genetic Toxicology Division
Health Effects Research Laboratory
Research Triangle Park, NC 27711

This study was conducted
in cooperation with
Northrop Services, Inc. - Environmental Sciences
Research Triangle Park, NC 27709

HEALTH EFFECTS RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
RESEARCH TRIANGLE PARK, NC 27711

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16. ABSTRACT

The overall objectives of this compilation are 1) to serve as a reference for workers in the genetic toxicology field, 2) as a starting point for creation of reference protocols for those who need precise Ames test protocols, and 3) as a guide to understanding variations in test results. This report, the result of an informal survey conducted by the U.S. Environmental Protection Agency, included laboratories that use the Ames test routinely. Protocols were requested from 46 laboratories included in a list supplied by Dr. Mike Shelby of the National Institute of Environmental Health Sciences. Twenty-five laboratories actually submitted protocols.

This publication is a simple compilation of the submitted protocols and cover letters. Any information not pertaining directly to the Ames test has been omitted and so noted on the cover sheet for each protoxol.

The purpose of this document is not to make a comment on the competence or performance of any laboratory. It is important to remember that some laboratories may have Standard Operating Procedures that are more detailed than submitted protocols. Some laboratories use the protocol by Ames et al. as the main procedure and have only sent their modifications. However, the compilers do feel that this report is a useful reference. It can be a guide for writing better protocols and it can give a better understanding of the variations of results from different laboratories.

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a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

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The Health Effects Research Laboratory, Research Triangle Park, North Carolina conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis, and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and

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surveillance of persons having suffered imminent and substantial endangerment of their health.

The reason for this compilation of 25 protocols for Salmonella mutagenicity testing is three-fold. It serves as a reference for genetic toxicologists, as a starting point for creation of reference protocols for those who need precise Ames test protocols, and as a guide in understanding variations in test results.

F. G. Hueter, Ph.D.
Director
Health Effects Research Laboratory

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ABSTRACT

The overall objectives of this compilation are 1) to serve as a reference for workers in the genetic toxicology field, 2) as a starting point for creation of reference protocols for those who need precise Ames test protocols, and 3) as a guide to understanding variations in test results. This report, the result of an informal survey conducted by the U.S. Environmental Protection Agency, included laboratories that use the Ames test routinely. Protocols were requested from 46 laboratories included in a list supplied by Dr. Mike Shelby of the National Institute of Environmental Health Sciences. Twenty-five laboratories actually submitted protocols.

This publication is a simple compilation of the submitted protocols and cover letters. Any information not pertaining directly to the Ames test has been omitted and so noted on the cover sheet for each protocol.

The purpose of this document is not to make a comment on the competence or performance of any laboratory. It is important to remember that some laboratories may have Standard Operating Procedures that are more detailed than submitted protocols. Some laboratories use the protocol by Ames et al. as the main procedure and have only sent their modifications. However, the compilers do feel that this report is a useful reference. It can be a guide for writing better protocols, and it can give a better understanding of the

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variations of results from different laboratories. A companion publication will also be available. It is a tabulating qualitative summary and comparison of submitted protocols.

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ACKNOWLEDGMENTS

The cooperation of the participating laboratories in submitting their protocols is gratefully acknowledged. In addition, the authors want to thank Ms. Leslie Silkworth, Northrop Services, Inc., Research Triangle Park, NC for editorial assistance.

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SECTION 1

INTRODUCTION

Bacterial tests for mutagenicity have been available for approximately 30 years. In 1951, Demerec et al. found that 19 out of 31 chemicals tested were mutagenic using an *Escherichia coli* reverse mutation system. After modifying the mutation system described by Demerec, Szybalski (1958) tested 431 substances for mutagenicity. Recognizing the inadequacies of the streptomycin resistance system, Ames (1971) published the methods for a bacterial system using a histidine-requiring mutant of *Salmonella typhimurium*. Malling (1971) was the first to merge a mammalian metabolizing system with a bacterial system in order to demonstrate the mutagenicity of dimethylnitrosamine.

In 1975, Ames et al. published a detailed protocol that incorporated the use of a mammalian metabolizing system thereby establishing the "Ames test" as a routine screening system for mutagenicity and potential carcinogenicity. Although the 1975 paper by Ames et al. provided a highly detailed protocol, many laboratories have introduced a variety of changes or additions. Also, summaries of international meetings have been published (Mattern and Greim, 1977; Seiler et al., 1979; de Serres and Shelby, 1979a; de Serres and Shelby, 1979b; Stich and San, 1979).

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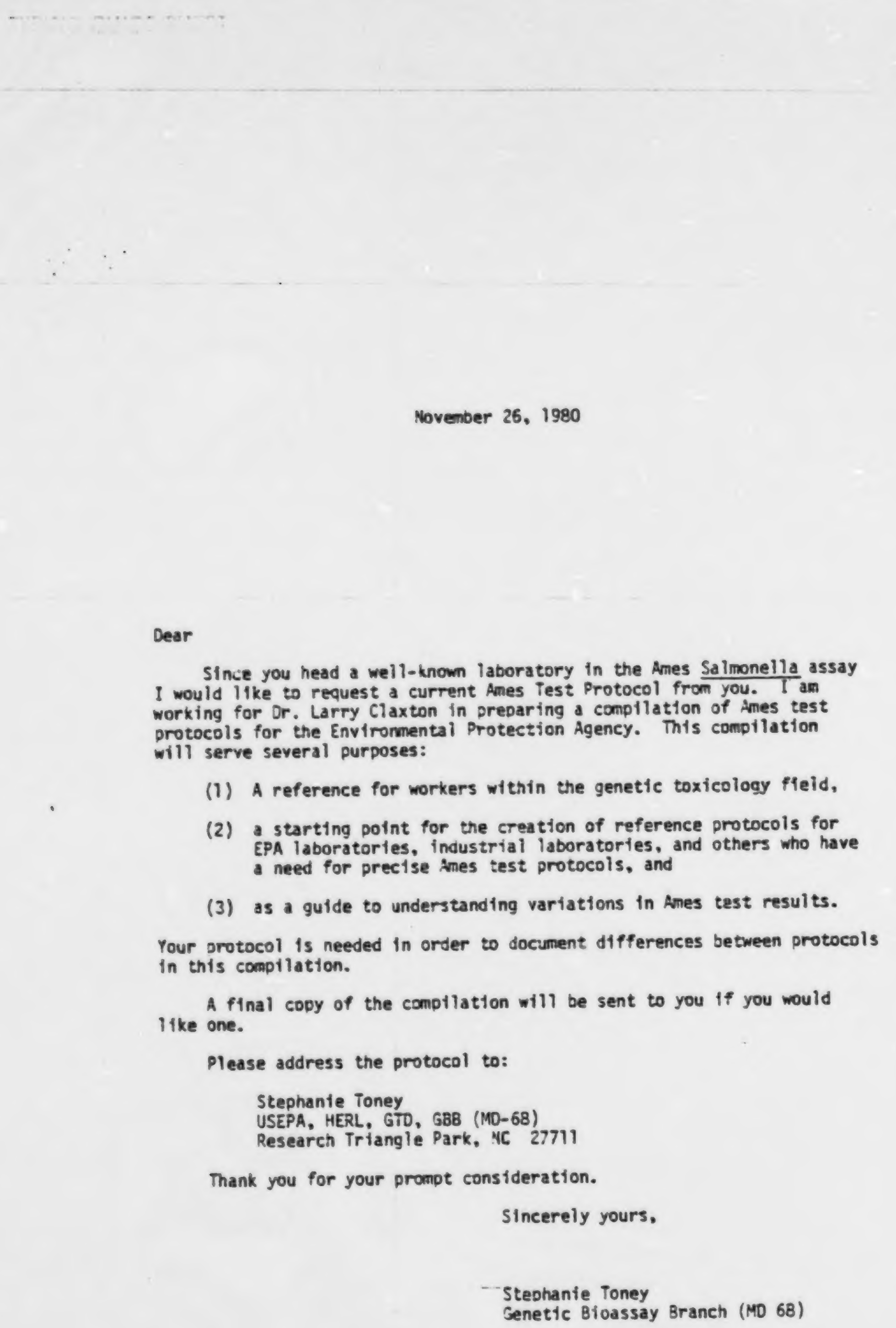
Since so many researchers and meetings have published suggested alterations and additions to the original protocol, the U.S. Environmental Protection Agency (EPA) decided to conduct an informal survey of laboratories that use the Ames test. A list of laboratories performing the Ames test in a routine manner was obtained from Dr. Mike Shelby at the National Institute of Environmental Health Sciences, Research Triangle Park, NC. An initial letter (see Figure 1) was sent to 46 laboratories included in this list. A follow-up letter (see Figure 2) was sent to the laboratories who had not responded within a one-month interval. As a result, 33 laboratories responded to the request. The responses included 6 laboratories that no longer performed the Ames test, 1 that had closed, 23 that did perform the test and submitted protocols, and 3 that performed the test but elected to restrict the usage of their protocols. In addition, two EPA laboratory protocols are included within this compilation. A list of names and addresses of the laboratories that participated by sending protocols is found in Table 1.

This publication is a simple compilation of the protocols and cover letters submitted. Any submitted information not pertaining directly to the Ames *Salmonella typhimurium* plate incorporation test have been omitted; however, any omissions are indicated at the beginning of each protocol. The compilers and EPA understand that these protocols may not accurately reflect the competence or performance of any laboratory. Some laboratories may maintain Standard Operating Procedures (SOPs) that are more detailed than the submitted protocols, and some may have modified or expanded their procedures since submission of this document. However, the compilers do feel that many



November 26, 1980

Dear

Since you head a well-known laboratory in the Ames Salmonella assay I would like to request a current Ames Test Protocol from you. I am working for Dr. Larry Claxton in preparing a compilation of Ames test protocols for the Environmental Protection Agency. This compilation will serve several purposes:

(1) A reference for workers within the genetic toxicology field,

(2) a starting point for the creation of reference protocols for EPA laboratories, industrial laboratories, and others who have a need for precise Ames test protocols, and

(3) as a guide to understanding variations in Ames test results.

Your protocol is needed in order to document differences between protocols in this compilation.

A final copy of the compilation will be sent to you if you would like one.

Please address the protocol to:

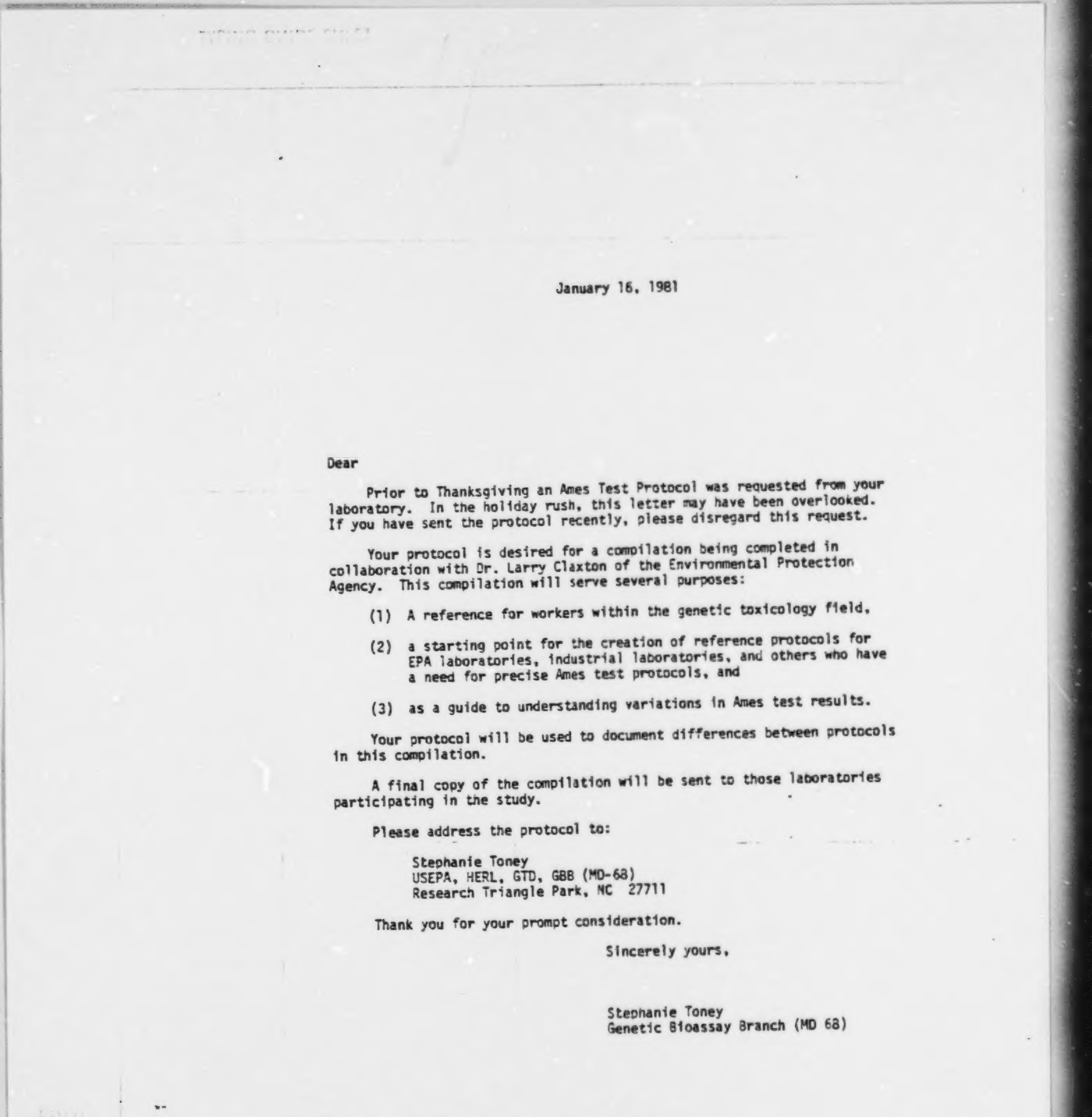
Stephanie Toney
USEPA, HERL, GTD, GBB (MD-68)
Research Triangle Park, NC 27711

Thank you for your prompt consideration.

Sincerely yours,

Stephanie Toney
Genetic Bioassay Branch (MD 68)

Figure 1. Initial letter of request.



January 16, 1981

Dear

Prior to Thanksgiving an Ames Test Protocol was requested from your laboratory. In the holiday rush, this letter may have been overlooked. If you have sent the protocol recently, please disregard this request.

Your protocol is desired for a compilation being completed in collaboration with Dr. Larry Claxton of the Environmental Protection Agency. This compilation will serve several purposes:

(1) A reference for workers within the genetic toxicology field,

(2) a starting point for the creation of reference protocols for EPA laboratories, industrial laboratories, and others who have a need for precise Ames test protocols, and

(3) as a guide to understanding variations in Ames test results.

Your protocol will be used to document differences between protocols in this compilation.

A final copy of the compilation will be sent to those laboratories participating in the study.

Please address the protocol to:

Stephanie Toney
USEPA, HERL, GTD, GBB (MD-68)
Research Triangle Park, NC 27711

Thank you for your prompt consideration.

Sincerely yours,

Stephanie Toney
Genetic Bioassay Branch (MD 68)

Figure 2. Follow-up letter of request.

TABLE 1. NAMES AND ADDRESSES OF PARTICIPATING LABORATORIES

Dr. Andrew Sivak
Arthur D. Little, Inc.
25 Acorn Park
Cambridge, MA 02140

Dr. James P. Crowley
Battelle Columbus Laboratories
505 King Ave.
Columbus, OH 43201

Dr. Douglas M. Hanson
Bioassay Systems Corporation
225 Wildwood Ave.
Woburn, MA 01801

Dr. Clyde R. Goodheart
Biolabs, Inc.
2910 MacArthur Blvd.
Northbrook, IL 60062

Dr. Rae E. Drazin
Bio-Technics Laboratories, Inc.
1133 Crenshaw Blvd.
Los Angeles, CA 90019

Dr. William T. Speck
Case Western Reserve University
Department of Pediatrics
2103 Adelbert Rd.
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Dr. Steve R. Haworth
EG & G Mason Research Institute
1530 E. Jefferson St.
Rockville, MD 20852

Dr. Larry D. Claxton
Genetic Toxicology Division
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711

Dr. Vincent F. Simmon
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6110 Executive Blvd.
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Rockville, MD 20852

(Continued)

TABLE 1. (Continued)

Ms. Nancy E. McCarroll
Hazleton Laboratories America, Inc.
9200 Leesburg Tnpk.
Vienna, VA 22180

Dr. George C. Lavelle
Hill Top Research, Inc.
Department of Toxicology
Miamiville, OH 45147

Mr. Peter W. Barbera
IIT Research Institute
Life Sciences Division
10 West 35th St.
Chicago, IL 60616

Ms. Shirley Louie
Jefferson Professional Services
P.O. Box 3397
Little Rock, AR 72203

Dr. Andrew M. Tometsko
Litron Laboratories, Ltd.
1351 Mount Hope Ave.
Rochester, NY 14620

Dr. David Brusick
Litton Bionetics, Inc.
5516 Nicholson Ln.
Kensington, MD 20795

Dr. Carol L. Richardson
Meloy Laboratories, Inc.
6715 Electronic Dr.
Springfield, VA 22151

Mr. Andrew M. Losikoff
Microbiological Associates
5221 River Rd.
Bethesda, MD 20016

Ms. JoAnne Gridley
Monsanto Research Corp.
1515 Nicholas Rd.
Dayton Laboratory
Dayton, OH 45407

(Continued)

TABLE 1. (Continued)

Dr. John E. Preston
U.S.E.P.A. - NEIC
Bldg. 53
P.O. Box 25227
Denver, CO 80225

Dr. Bruce C. Casto
Northrop Services, Inc.
P.O. Box 12313
Research Triangle Park, NC 27709

Dr. Robert A. Finch
Raltech Scientific Services
A Division of Ralston Purina Co.
P.O. Box 7545
Madison, WI 53707

Mr. Thomas J. Hughes
Research Triangle Institute
P.O. Box 12194
Research Triangle Park, NC 27709

Dr. Nathan D. Greene
Southwest Foundation for Research
and Education
P.O. Box 28147
San Antonio, TX 78284

Dr. David C.L. Jones
SRI International
333 Ravenswood Ave.
Menlo Park, CA 94025

Ms. Melanie Baltezore
ES Unilab Research, Inc.
2800 Seventh St.
Berkeley, CA 94710

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will find this report a useful reference, a guide for writing better protocols, and a guide to understanding why variations in results from various laboratories exist. A companion volume tabulates and discusses the similarities and differences between the submitted protocols.

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SECTION 2

DESCRIPTION OF THE AMES *Salmonella typhimurium* PLATE INCORPORATION TEST

The Ames test is a short-term bioassay for mutagenicity testing. The advantages of this test are the speed with which results can be obtained, usually three to four days, and the relative low cost. The Ames test is used for testing both pure chemicals (cyclophosphamide and 6-mercaptopurine) and complex mixtures including mobile source emissions (diesel and gasoline) and comparative source emissions (coke oven, roofing tar, and cigarette smoke condensate).

The Ames test basically involves taking a given sample and adding it to a strain of *Salmonella* in an agar overlay tube that is then plated on minimal media (see Figure 3). The sample is routinely tested at several dose levels with five Ames strains of *Salmonella*, with and without metabolic activation. The dose levels are plated usually in duplicate or triplicate. This plating involves approximately 120 plates/sample excluding the control plates.

After the bacteria, test compound, and possibly metabolic activation are added to the overlay tube, the contents are gently mixed and plated out on Vogel-Bonner minimal media plates. These plates are incubated for three days at 37°C in the dark. After three days the plates are scored for mutants.

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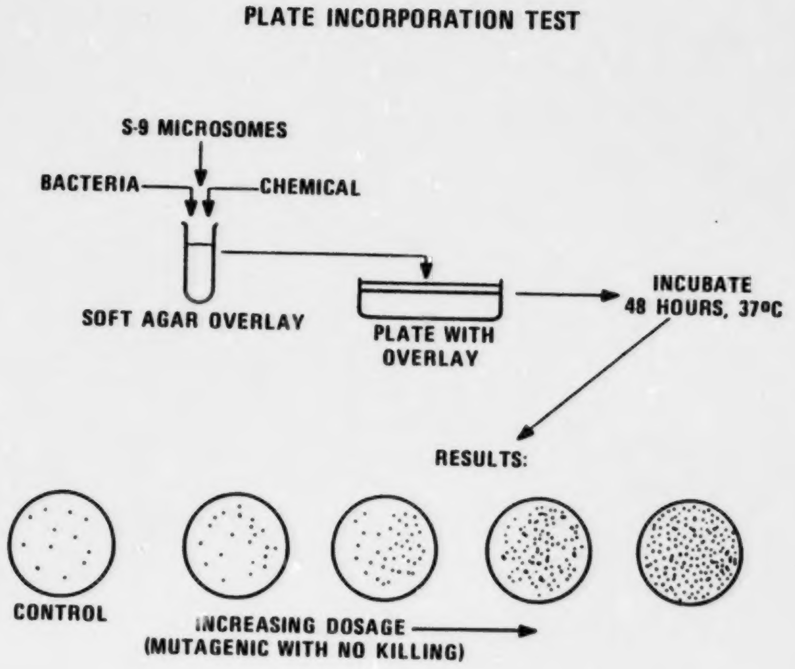

Figure 3. Schematic for plate incorporation test.

Five Ames strains are in widespread use--TA1537, TA1538 and its derivative TA98, and TA1535 and its derivative TA100. These strains are all histidine-dependent mutants and revert to wild type in the Ames test. Two of the strains detect frameshift mutations (TA1537 and TA1538), and one strain detects base-pair substitutions (TA1535). Strains TA98 and TA100 are less specific as to the type of mutants they detect. Strains TA98 and TA100 both contain an R factor plasmid that increases the sensitivity of these strains. All five strains have a rfa mutation creating a deficiency in the lipopolysaccharide cell wall therefore increasing permeability to macromolecules. The strains all have a *uvr* B mutation that decreases genetic repair ability.

The mammalian metabolic activation system (S-9) is added to the overlay to identify compounds that require the metabolic activation mechanisms of mammals not found in bacteria. The mammalian metabolic activation system permits metabolites of the test compound to be tested for mutagenicity. Ames' activation system generally consists of a 9000 x g supernatant of Aroclor-1254-induced rat liver homogenate.

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Szybalski, Waclaw. 1958. Special microbiological systems. II. Observations on chemical mutagenesis in microorganisms. N.Y. Acad. Sci. Anal. 76:475-489.

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Malling, H.V. 1971. Dimethylnitrosamine: formation of mutagenic compounds by interaction with mouse liver microsomes. Mutat. Res. 13:425-429.

Ames, B.N., Joyce McCann, and Edith Yamasaki. 1975. Methods for detecting carcinogens and mutagens with the *Salmonella*/Mammalian-Microsome Mutagenicity Test. Mutat. Res. 31:347-364.

Mattern, I.E., and Helmut Greim. 1978. Report of a workshop on bacterial *in vitro* mutagenicity test systems. Mutat. Res. 53:369-378.

de Serres, Frederick J., and Michael D. Shelby. 1979. Recommendations on data production and analysis using the *Salmonella*/microsome mutagenicity assay. Mutat. Res. 64:159-165.

de Serres, Frederick J., and Michael D. Shelby. 1979. The Salmonella mutagenicity assay: recommendations. Science 203:563-565.

Seiler, J.P., I.E. Mattern, M.H.L. Green, and D. Anderson. 1980. Second European workshop on bacterial *in vitro* mutagenicity test systems. Mutat. Res. 74:71-75.

Stich, H.F., and R.H.C. San. 1980. International workshop on short-term tests for chemical carcinogens. Mutat. Res. 74:411-414.

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APPENDIX A

Laboratory: Arthur D. Little
Acorn Park
Cambridge, MA 02140

Submitter: Dr. Andrew Sivak
Vice President
Biomedical Sciences
(617)864-5770

Date of submission: February 13, 1981

Omissions: None

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Arthur D Little, Inc. ACORN PARK · CAMBRIDGE, MA 02140 · (617) 864-5770 · TELEX 921436

February 13, 1981

Ms. Stephanie Toney
Genetic Bioassay Branch (MD 68)
United States Environmental Protection Agency
Health Effects Research Laboratory
Research Triangle Park
North Carolina 27711

Dear Ms. Toney:

In response to your letter of January 16, 1981, requesting our Ames assay protocol, for routine use especially for environmental samples, we presently utlize the procedure described in the IERL-RTP Procedures Manual: Level 1 - Environmental Assessment Biological Tests for Pilot Studies. EPA - 600/7-77-043, April, 1977. Since the Agency already has the details of this protocol, it does not seem to be necessary to provide a duplicate to you.

We have performed a number of special studies, but the design of these was done in consultation with our clients to address specific needs.

We would be pleased to participate in any on-going review of protocols that could contribute to a better understanding of interpretation and use of data from the Salmonella mutation assay.

Sincerely yours,

Andrew Sivak, Ph.D.
Vice President
Bio/Medical Sciences

AS:km

CAMBRIDGE, MASSACHUSETTS
ATHENS BRUSSELS LONDON PARIS RIO DE JANEIRO SAN FRANCISCO TORONTO WASHINGTON WIESBADEN

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3.2.5 Range-finding and Definitive Tests

For all the following biotests, it is usually desirable to conduct a range-finding or screening test to determine the concentrations that should be used in the definitive test, unless the approximate toxicity of an effluent is already known or unless the sample size is limiting. This preliminary testing can save both time and money. Range-finding tests should be conducted using three to five widely spaced effluent concentrations (covering the entire range of 0 to 100 percent effluent, if possible). Conditions for the range-finding and definitive tests should be kept as similar as possible; the greater the similarity between the two tests, the more useful the results of the range-finding tests will be.

3.3 HEALTH EFFECTS TESTS

3.3.1 Salmonella/Microsome Mutagenesis Assay (Ames') (Reference 5)

The Ames assay is based on the property of selected Salmonella typhimurium mutants to revert from a histidine requiring state to prototrophy due to exposure to various classes of mutagens. The test can detect to nanogram quantities of mutagens and has been adapted to mimic some mammalian metabolic processes by the addition of a mammalian liver 9,000 $\overline{G}$ microsomal fraction (S-9). The test will be used as a primary screen to determine the mutagenic activity of complex mixtures or component fractions. It has recently been demonstrated that most carcinogens act as mutagens. In extensive testing, the Ames' assay has demonstrated 90 percent accuracy in detecting known carcinogens as mutagens. Certain known carcinogens are negative in the test (e.g., asbestos, metals) or weakly positive. False positives are also known which are mutagenic in the Ames' system but which have been found to be noncarcinogenic in mammals. Continued improvement of the present bacterial strains, addition of new strains, and re-evaluation of the conventional animal carcinogenesis data should reduce this level of error in the near future. The following is intended as a general description of the test; for detailed protocol see the published method. (Reference 5)

3.3.1.1 Method Description

Materials. The species of bacteria to be used are the Salmonella typhimurium tester strains developed by Dr. Bruce Ames. Specifically, these are TA-1535, TA-1537, TA-1538, TA-98, and TA-100. The tester strains

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are histidine deficient variants and are used to detect frameshift reverse mutations (TA-1537, -98, and -1538) or base pair substitutions (TA-1535 and -100) as indicated by reversion to prototrophy. For general screening, strain TA-1538 may be omitted.

Liver Microsome Preparations. The activation system for mutagenesis screening consists of Aroclor 1254 induced S-9 fraction derived from rat livers. Rats should be males (Sprague-Dawley) about 200 g each. Induction is accomplished by a single intraperitoneal injection of Aroclor 1254 (diluted in corn oil to 200 mg/ml) into each rat five days before sacrifice at a dosage of 0.5 mg/g of body weight. All rats are deprived of food (not water) 12 hours before sacrifice. The rats are then stunned by a blow on the head and decapitated. The following steps are carried out at 4 C using cold sterile solutions and glassware.

The livers (10-15 g) are aseptically removed from the rats and placed into a cold preweighed beaker containing 10-15 ml of 0.15M KCl. After the livers are washed and weighed in this beaker they are removed with forceps to a second beaker containing 3 ml of the KCl solution per gram of wet liver weight. The livers are then minced with sterile scissors, transferred to a chilled glass homogenizing tube, and homogenized in an ice bath by passing a low speed motor driven pestle through the livers a maximum of three times. The chilled homogenates are then placed into centrifuge tubes and centrifuged for 10 minutes at 9000 G at 4 C. The resulting supernatant is decanted, transferred in 2-ml amounts to small storage tubes, quickly frozen in dry ice, and stored at -80 C in a low temperature freezer. This supernatant is known as the S-9 fraction. Sufficient S-9 for use each day is thawed at room temperature and kept on ice before and during use. The extent of bacterial contamination of the S-9 fraction should be determined. The S-9 mix may be filter sterilized (0.45 μm porosity filter) if required.

Metabolic Activation Mixture. The S-9 microsomal mix is prepared according to the recommendations of Ames. The mix contains per ml: S-9 (0.04-0.1 ml), $MgCl_2$ (8 μmoles), glucose-6-phosphate (5 μmoles), nicotinamide adenine dinucleotide phosphate (NADP) (4 μmoles), and sodium phosphate, pH 7.4 (100 μmoles). Stock solutions of NADP (0.1 M) and glucose-6-phosphate (1.0M) are prepared with sterile water, in appropriate amounts, and maintained

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at -20 C. The stock salt solution (0.4M $MgCl_2$ and 1.65 KCl) and phosphate buffer (0.2M pH, 7.4) are prepared, autoclaved, and refrigerated. The S-9 mix is prepared fresh each day and is maintained on ice before and during use. The activity of the S-9 preparation should be standardized by measurement of its aryl hydrocarbon hydroxylase activity and by determination of its total protein content.

Bacteriological Media. The minimal-glucose agar medium for histidine requiring strains used in mutagenesis assays is a 1.5 percent Bacto-Difco agar in Vogel-Bonner Medium E with 2 percent glucose. Top agar (0.6 percent Difco agar, 0.5 percent NaCl) contains a trace of histidine and biotin to permit the bacteria to undergo several divisions. The top agar medium used in the (optional) toxicity assays is the same medium fortified with 30 mg/ml of histidine. Fewer bacteria (about 10^2 cells/plate) are used in the toxicity test.

Solvents and Positive Control Chemicals. The solvent for all stock control chemicals is a spectrophotometric grade dimethyl sulfoxide. The following compounds are examples of compounds used for positive control assays. (Ref. 5) Others may be substituted.

Indicator Strain	Nonactivation Assays	Activation Assays
TA 1535	MNNG (N-Methyl-N'-nitro-N-nitrosoguanidine)	2-anthramine
TA 1537	9-aminoacridine*	2-anthramine
TA 98	Daunomycin	2-anthramine
TA 100	MMS (Methyl methanesulfonate)	2-anthramine

*9-aminoacridine is dissolved in ethanol.

Methods. Each sample should undergo a mutagenesis test, an optional toxicity test, a positive control test, and a sterility control test. The plate incorporation test is recommended for routine use. The cells used for the toxicity and mutagenesis determinations are derived simultaneously from the same bacterial population. Viability in the toxicity assay will be used to adjust counts of mutant colonies by the estimated proportion of the surviving population.

In the plate incorporation assay the sample under investigation is added directly to the molten top agar and is poured onto the plate along with the indicator test organism and the liver S-9 activation system.

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It is recommended that the plate incorporation test be performed (preferably in duplicate) initially at 0.01, 0.1, 1 and 10 mg/plate, and that repeat studies be performed over a narrower concentration range showing positive results in the initial test (up to a maximum concentration of 20 mg/plate).

The test compound is evaluated with all tester strains in the presence and absence of the liver S-9 activation system. Liquid samples are assayed similarly with the maximum sample volume being about one ml. Controls are included at all times and consist of a control for the spontaneous reversion rate for each tester strain where the mutagen is omitted, a sterility check of the mutagen solution, and a positive control consisting of compounds which both do and do not require metabolic activation.

The number of spontaneous reversions/plate should be within the following limits or those specified in Reference 5.

Strain	Acceptable Spontaneous Revertants/Plate
TA-1535	20 ± 10
TA-1537	15 ± 10
TA-98	50 ± 25
TA-100	150 ± 75

A sample will be considered negative if the number of induced revertants obtained as compared to the spontaneous revertants is less than two-fold.

Compounds which indicate positive reversions (at least a two-fold increase in reversion rate) will be examined by preparing dose response curves with the tester strain(s) indicating high reversion rates. The dose response curve will be performed in duplicate at five concentrations of the sample found to be mutagenic. A two-fold increase in reversion rate with evidence of increasing reversion with increasing sample concentration shall constitute a positive test. In addition, the dose response curve should be reproducible.

Sterility Controls. Each bioassay includes a sterility control check for each test component. These include the S-9 mix, the compound under test, and the solvent vehicle.

Phenotype Monitoring. After the number of colonies on each plate is counted, occasional samples (for example, 10) of the revertant colonies are picked from each plate exhibiting a positive response. These colonies are restreaked onto minimal agar to confirm if they are revertants or potential phenocopies.

3.3.1.2 Results

The plate incorporation mutagenesis/toxicity test provides an indication of both mutagenicity and toxicity of the sample. Toxicity is evidenced by a reduction in the number of colonies (viability) of bacteria. Counts of mutant colonies in the plate incorporation mutagenesis test are adjusted by multiplying average counts by 1/viability. Relative mutagenic activity is derived from the adjusted average colony counts by representing the control plate count (vehicle only) as 1.00 and then converting proportionately the adjusted average counts from the plates which contain test sample.

3.3.2 Cytotoxicity Assays (References 6-11)

Cytotoxicity assays employ mammalian cells in culture to measure quantitatively cellular metabolic impairment and death resulting from exposure *in vitro* to soluble and particulate toxicants. Mammalian cells derived from various tissues and organs can be maintained as short term primary cultures or in some cases, as continuous cell strains or lines. Primary cell cultures exhibit many of the metabolic and functional attributes of the original tissues, some of which may be lost with prolonged passage in culture. Both types of systems have been employed effectively in cytotoxicity screening of, for example, pharmaceuticals (especially antibiotics and anti-tumor agents), implantable medical polymers and mineral crystals and fibers. More recently, such systems have been applied in evaluating the relative cellular toxicity of hazardous metallic salts (Ref. 6) and industrial air particulates (Ref. 7). As compared to conventional whole animal tests for acute toxicity, cytotoxicity assays are more rapid, less costly and require significantly less sample. The tests can provide useful information about the relative cellular toxicity of unknown samples. However, it should be understood that because the assays employ isolated cells and not intact animals, they can provide only preliminary and imprecise information about the ultimate health hazards of toxic chemicals.

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APPENDIX B

Laboratory: Battelle Columbus Laboratories
505 King Ave.
Columbus, OH 43201

Submitter: Dr. James P. Crowley
Associate Manager
Biomedical Sciences Section
(614)424-7622

Date of submission: December 2, 1980

Omissions: CHO-Cytotoxicity Assay
Rabbit Alveolar Macrophage Assay

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Battelle
Columbus Laboratories
505 King Avenue
Columbus, Ohio 43201
Telephone (614) 424-6424
Telex 24-5451

December 2, 1980

Stephanie Toney
USEPA, HERL, GTD, GBB (MD-68)
Research Triangle Park, NC 27711

Dear Ms. Toney:

Enclosed are the protocols and other related information which you requested. If you have any further questions, please contact me at (614) 424-7622 or Dr. June Carver at (614) 424-4605.

Very truly yours,

James P. Crowley, Ph.D.
Associate Manager
Biomedical Sciences Section

JPC/wk

Enclosures

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TASK I - AMES BACTERIAL MUTAGENESIS BIOASSAY

INTRODUCTION

The Ames bacterial mutagenicity test is a bioassay designed to detect potential mutagens by means of a special set of five *Salmonella typhimurium* strains developed by Dr. Bruce Ames. Specifically these are TA-1535, TA-1537, TA-1538, TA-98, and TA-100. These strains are all histidine autotrophs by virtue of mutations in the histidine operon. When these histidine-dependent cells are grown on a minimal media deficient in histidine, only those cells that revert to histidine independent (his$^+$) are able to grow (mutants). The spontaneous mutation frequency of each strain is relatively constant, but when a mutagen is added to the agar the mutation frequency is increased from three to hundreds of times.

In addition to having mutations in the histidine operon, all the indicator strains have mutations in the lipopolysaccharide coat ($\underline{rfa^-}$) and deletions that cover a gene involved in the repair of uv damage ($\underline{uvrB^-}$). The $\underline{rfa^-}$ mutation makes the strains more permeable to large molecules, therby increasing their sensitivity to these molecules. The $\underline{uvrB^-}$ mutation decreases repair of some types of chemically damaged DNA and thereby enhances sensitivity to some mutagenic chemicals.

Strain TA-1535 is reverted to histidine prototrophy (his$^+$) by many mutagens that cause base-pair substitutions. Similarly strains TA-1537 and -1538 are able to detect various kinds of frameshift mutagens. Tester strains TA-100 and TA-98 are derived from strains TA-1535 and -1538, respectively, by the introduction of the R factor plasmid pkM101. The introduction of this plasmid, which confers ampicillin resistance to the strains, greatly enhances the sensitivity of the respective strains to some base-pair and frameshift mutagens. The presence of this plasmid also makes strain TA-100 sensitive to some frameshift mutagens.

The assay has been adapted for use in detecting compounds which may be potential mutagens. It has been documented that most

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compounds that act as carcinogens in mammals also act as mutagens in bacterial systems. However, a significant percentage of known carcinogenic compounds are not active carcinogens in the parent form but require enzymatic alteration to an active moiety. Mammalian microsomal hydroxylase systems are responsible for this activation. Since these specific bacteria do not have the mammalian microsomal enzyme system, mammalian liver homogenates are added to the system to activate the non-mutagenic parental compounds to possible mutagens.

MATERIALS

Described within this section are the materials and preliminary steps in the preparation of the liver microsomes and bacteriological plating media which are used in toxicological and mutagenicity studies of compounds using the Ames standard *Salmonella* tester strains.

Liver Microsome Preparations

The activation system for mutagenesis screening consists of Arochlor 1254 induced microsomes derived from rat livers. Induction is accomplished by a single intraperitoneal injection of Arochlor (diluted 200 mg/ml of corn oil) into adult male rats weighing about 200 g each, at a dosage of 0.5 mg/g of body weight, 5 days before sacrifice. The rats are deprived of food 24 hours before sacrifice. The rats are stunned by a blow on the head and decapitated.

The livers are aseptically removed from the rats and placed into a cold preweighed beaker containing 10 ml of 0.5M KCL. After the livers are swirled in this beaker they are removed with forceps to a second beaker containing 3 ml of the KCL solution per gram of wet liver weight. The livers are then minced with sterile scissors, transferred to a chilled glass homogenizing tube and homogenized by passing a low-speed motor driven pestle through the livers a maximum of four times. The homogenates are then placed in cold centrifuge tubes and centrifuged for 10 minutes at 9,000 G at 4°C. The resulting supernatant is decanted,

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aliquoted in 3 ml amounts to small culture tubes, quickly frozen in dry ice, and stored at -80°C in a Revco freezer. Sufficient microsomes for use each day are thawed at room temperature and kept on ice before and during use.

Microsomal Mix

The S-9 microsomal mix is prepared according to the recommendations of Ames. The mix contains per ml: S-9 (0.10 ml), $MgCl_2$ (8 µmoles), KCL (33 µmoles), glucose-6-phosphate (5 µmoles), NADP (4 µmoles), and sodium phosphate, pH 7.4 (100 µmoles). Stock solutions of NADP (0.1M) and glucose-6-phosphate are prepared with sterile water, aliquoted in appropriate amounts, and maintained in a Revco freezer. The stock salt solutions are prepared, autoclaved, and refrigerated. The S-9 mix is prepared fresh daily and is maintained on ice before and during use. Total protein content are routinely determined. For this study, the activity of the S-9 preparations will be standardized by (a) aryl hydrocarbon hydroxylase activity, and (b) mutagenic response to 2-AF and BaP; if variability occurs among S-9 preparations, the standardization of choice would be based on equivalent response to known mutagens.

Bacteria

The *Salmonella* tester strains were obtained directly from Ames and stock solutions of the strains have been stored in the Revco freezer. At monthly intervals, new bacterial isolates are obtained from this stock supply. Each clonal culture is checked for confirmation of biochemical activity and spontaneous reversion rate. The cultures which conform to the specifications of Ames are streak isolated and used as master cultures. These master cultures are used as the origin of weekly preparations of working broth cultures. All broth cultures are nutrient broth (Difco) supplumented with 0.5 percent NaCl. The bacterial cultures to be used for an assay will be prepared by inoculating 0.1 ml of each strain into 10 ml of nutrient broth and incubating

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the culture in a water bath shaker for 16-20 hours. The cultures used for an assay will have been incubated the night before the assay and will be kept on ice during the process of the assay.

Bacteriological Media

The selective basal medium for all histidine requiring strains used in mutagenesis assays is a 1.5 percent Bacto-Difco agar in Vogel-Bonner Medium E with 2 percent glucose. The top agar (0.6 percent Difco agar, 0.5 percent NaCl) is prepared in 200 ml aliquots, autoclaved, and stored at room temperature. Before use in mutagenesis assays, the agar is melted and 20 ml of a sterile solution of 0.5 mM λ-histidine, HCl, and 0.5 mM biotin is added to the molten top agar and mixed thoroughly. The top agar is then aliquoted in 2 ml amounts in sterile culture tubes and maintained at 45°C in a water bath before use.

Microsomal Optimization. Plate incorporation assays have been conducted in duplicate using tester strain TA-98. Test substances included benzo(a)pyrene (1 μg BAP/Plate) and 2 amino fluorene (5 μg 2AF/plate). The test chemical concentration remained constant and the amount of S-9 mix added per plate was varied. Control plates contained no S-9 activation system.

Revertants Per Plate

ml of S-9 mix	BAP	2AF
0	26	35
0.1	112	1345
0.2	266	1379
0.3	207	857
0.4	125	534
0.5	102	415
0.6	89	349
0.7	76	246
0.8	60	238

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Throughout the assays conducted in this investigation, plates containing the activation system (designated +) will contain 0.2 ml of the 10 percent microsomal enzyme mixture.

Solvents and Positive Control Chemicals

The purpose of the positive control assay is to confirm the performance of the test components with and without microsomal activation and to provide a standard against which any activity of the test substances may be compared. The solvents for all stock control chemicals are a spectrophotometric grade dimethyl sulfoxide. The following compounds are recommended for positive control assays.

Indicator Strain	Nonactivation Assays	Activation Assays
TA-1535	sodium azide	2-aminoanthracene
TA-1537	9-aminoacridine	2-aminoanthracene
TA-1538	2-nitrofluorene	2-aminoanthracene
TA-98	2-nitrofluorene	2-aminoanthracene
TA-100	sodium azide	2-aminoanthracene

Sterility Controls

Each bioassay will include a sterility control check for each test compound. These will include the S-9 mix, the compound under test, and the solvent vehicle.

GENERAL EXPERIMENTAL DESIGN

Preliminary Cytotoxicity Evaluation

Each of the samples under investigation will be examined by a preliminary plate incorporation assay. For this assay, dilutions of the test sample are added directly to the molten top agar and are poured along with the test organism. The test compound will be evaluated with

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only one of the tester strains (usually TA-98) since the toxicity response of all tester strains for a particular compound are so similar that the information obtained from a single strain is applicable to the other four. The evaluation will be conducted by incorporating a wide range of concentrations of the test compound into the top agar tubes containing the tester strain with subsequent pouring onto mutagenicity agar plates. The maximum amount of test material to be used in this preliminary evaluation will depend on the amount of samples available.

The toxicity of the test sample will then be determined by careful observation of the growth of the inoculated organisms in the top agar following incubation. If a compound is not toxic, the inoculated organisms can be observed with a dissecting microscope as a lawn of small colonies. Toxicity of a compound is determined as a function of the growth of this lawn. At concentrations of the compound where the background lawn is nonexistent, the compound will be declared to be cytotoxic. Based on previous experience with fly ash particulates, the dose of solid sample that can be practically applied is about 20 mg/plate.

Bacterial Mutagenicity

After the preliminary toxicity assay is completed, the concentrations of the test sample will be chosen for the mutagenicity assay. Depending upon sample availability, the highest concentration to be utilized in the mutagenicity test will be (a) that which has demonstrated a slightly toxic response by virtue of having decreased population of inoculated organisms in the preliminary assay but which did not demonstrate a fully toxic response, or (b) the maximum practical dose, i.e., $\leq$20 mg/plate.

Bacterial mutagenicity evaluations will be made in duplicate against all five of the Ames tester strains for five dose levels over the range 0.01 to $\geq$10 μg/plate selected from the preliminary cytotoxicity data. A 0.1 ml aliquot of an overnight culture is added to the 2 ml of molten top agar supplemented with a trace of histidine and biotin.

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For nonactivation tests, the particulate sample will be suspended in glycerol, diluted 1:1 with H_2O, and will be added to the tubes and poured over the selective agar plates; based on previous experience with fly ash, this concentration of glycerol is not toxic to the *Salmonella* test strains and serves to hold the particulate matter in homogeneous suspension during dilutions and subsequent assay. In activation tests, a volume of the S-9 microsomal mix will be added to the top agar tubes after the selected concentrations of the test material have been added and just prior to pouring. The volume of the microsomal mix will have been previously determined to be optimum to elicit the highest possible response for a positive control mutagen. Normally this will be benzo[a]pyrene although optimization can be determined for any other selected positive control compound. Following solidification, the plates will be incubated for approximately 72 hours and the number of revertant colonies counted.

Application of particulates to the *Salmonella* plate incorporation test measures the aqueous solubility of a particle-bound mutagen and its subsequent diffusion through aqueous culture medium and agar to the target cell. A variety of chemical mutagen classes are not water-soluble and would not be detected by the whole sample assay. Therefore, the Battelle staff recommends that, where sample availability permits, the particles be extracted for 4 hours with DMSO and the resulting organic extract applied to the *Salmonella* bioassay per the above protocol. This will increase the validity of the *Salmonella* mutagenesis test results and greatly strengthen the credibility of negative mutation data.

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Phenotype Monitoring

After the number of colonies on each plate are counted, random samples of the revertant colonies will be picked from each plate exhibiting a positive response. These colonies will then be restreaked onto minimal agar to confirm if they are revertants or potential phenocopies. Generally, ten colonies will be picked from each plate for confirmation.

Criteria for Valid Test

The following criteria must be met before the results of a mutagenicity assay may be judged as positive or negative. The test must be repeated if any of these criteria are not met.

(1) The sterility check of the test components must yield less than two viable cells per plate.

(2) The positive controls must produce at least a threefold increase in the number of revertants over the average value of the respective negative control.

(3) The average number of spontaneous revertants (negative control values) must fall within the following limits:

Strain	Acceptable Range
TA-1535	20 ± 10
TA-1537	9 ± 10
TA-1538	24 ± 15
TA-98	40 ± 25
TA-100	150 ± 75

Criteria for a Positive Mutagenic Response

Because the procedures used to evaluate the mutagenicity of a test compound are semiquantative, the criteria used to determine positive effects are inherently subjective and are based primarily on a historical data base. The following criteria are used by the Battelle Laboratories.

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(1) If the solvent control value is within the normal range, a chemical mutagen must produce a positive response with the lowest increase equal to twice the solvent control.
(2) The positive response should be dose responsive, i.e., the dose response increase should start at approximately the solvent control value and the number of revertants should increase in direct proportion to the quantity of chemical being examined, and
(3) The dose response pattern must be reproducible in one or more additional assays. Repeat assays are dependent upon sample availability.

These criteria are not absolute and other extenuating factors enter into the final decision. As an example, TA-1535 and TA-100 were both derived from the same parental strain (G-46), likewise TA-1538 and TA-98 were derived from the same parental strain (D3052), thus there is a built-in redundancy in the microbial assay. However, the two strains of a set respond to the same mutagen and such a pattern is sought. If is also anticipated that if a given strain responds to a mutagen in nonactivation tests, it will generally do so in activation tests. The converse of this relationship is not expected. While there response patterns are not required for all mutagens, they are used to enhance the reliability of the evaluation decision. All data will be provided in tabular format, per the attached.

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FIGURE 3. GENERAL FORMAT FOR RECORDING MUTAGENICITY DATA

Date Sample Received ____________________
Sample ID Numbers____________________
Incubation Temperature____________________
Incubation Start____________________
Incubation End____________________
Investigator____________________

Tester Strain Used__________

Report Date____________________
Date Tested____________________
Date Reported____________________
Comments, i.e. Sol. Extrac. or Samp.
Pretreatment,etc.____________________
Calculations & Report Rec'd by____

Test Condition	Sample Type	Assay Amount (µg/plate)	Revertants or colonies on individual plates				Average		Adjusted Average Revertants		Index of Relative Mutagenicity	
			Number 1	Number 2	Repeat 3	Repeat 4	1 & 2	3 & 4	1 & 2	3 & 4	1 & 2	3 & 4
Nonactivation	Positive Control											
	Solvent Control											
	Sample Plate ID											
	Toxicity Test											
Induced Activation	Positive Control											
	Solvent Control											
	Sample Plate ID											
	Toxicity Test											

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APPENDIX C

Laboratory: Bioassay Systems Corporation
225 Wildwood Ave.
Woburn, MA 01801

Submitter: Dr. Doug Hanson

Date of submission: December 1980

Omissions: Salmonella Microtoxicity Assay
Preincubation Assay
Quality Assurance Manual

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Bioassay Systems

Standard Procedure

Section: Genetic Tox.
No.: 080-002
Effective: 10/22/79
Page 1 of 7
Approved: Kenneth S. Loveday

Replaces Original

Subject: Salmonella/microsome Mutagenesis Assay Test Protocol

A. PURPOSE

The Salmonella/microsome test is a simple and sensitive bacterial test for detecting chemical mutagens. Approximately 85% of the carcinogens tested to date have been detected as mutagens. This high number of potential carcinogens is the rationale for using the Salmonella/microsome test as one of the bioassays for screening for carcinogens.

In this test, compounds are assayed on minimal agar petri dishes with several specially constructed mutants of Salmonella typhimurium selected for sensitivity and specificity in being reverted from histidine requirement back to prototrophy by a wide variety of mutagens. To increase the scope of sensitivity of the test, a rat liver homogenate (S-9 fraction) is directly applied to the plates and thus the carcinogens requiring metabolic activation can also be detected.

B. CITATION

Ames, B. N., McCann, J., and Yamasaki, E. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Research 31:347-364, 1975.

C. MATERIALS

1. Stock solutions.

   a. 50X VB salts:

      i. Formula (add in order),

45°C distilled water	670 ml
$MgSO_4 \cdot 7H_2O$	10 g
Citric Acid $\cdot H_2O$	100 g
K_2HPO_4 (anhydrous)	500 g
$Na(NH_4)HPO_4 \cdot 4H_2O$	175 g

   b. 40% Glucose:

      50 ml of 40% glucose per liter of bottom agar. Prepare as much as practical, filter sterilize.

Bioassay Systems

Standard Procedure

Section: Genetic Tox.
No.: 080-002
Effective: 10/22/79
Page 2 of 7
Approved: Kenneth S Loveday

Replaces Original

Subject: Salmonella/microsome Mutagenesis Assay Test Protocol

c. Bottom Agar:

A large stock of bottom agar is suggested as 1 liter only makes about 34 base layer plates. One quantitative test requires about 150 plates. Bottom agar is 1.5% agar, i.e. 15 g agar per liter distilled water. Autoclave and store at room temperature in 500 ml aliquots. When preparing, the water has to be boiling to bring the powder into solution. The dispensing should also be done when the agar is still hot.

d. Top Agar:

Agar	6 g
NaCl	5 g
distilled water	1 liter

Dispense in 100 ml aliquots. Add 8 ml of distilled water and autoclave, store at 4°C.

e. Broth:

Nutrient broth	8 g
NaCl	5 g
distilled water	1 liter

Dispense in 5 ml aliquots and autoclave, store refrigerated.

f. Biotin and Histidine Stocks:

Biotin 5 mM (122.2 mg/100 ml)

Filter sterilize, store at room temperature. Heat gently before use if it precipitates. 100 ml will make 5,000 plates.

L-Histidine · HCl · H_2O 5 mM (104.9 mg/100 ml)

Filter sterilize, store in darkness (wrap the bottle in aluminum foil). 100 ml will make 5,000 plates.

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Bioassay Systems

Section: Genetic Tox.
No.: 080-002
Effective: 10/22/79
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Approved: Kenneth S. Loveday

Standard Procedure

Replaces Original

Subject: Salmonella/microsome Mutagenesis Assay Test Protocol

2. S-9 Mix Stocks.

a. Formula:

KCl	1.65 M (12.30 g)
$MgCl_2 \cdot 6H_2O$	0.40 M (81.32 g)
distilled water	100 ml

Autoclave and store refrigerated. 100 ml will make 5000 ml of S-9 mix.

Glucose-6-phosphate	1 M (7.05 g)
distilled water	25 ml

Filter sterilize and store at -20°C. 25 ml is good for 5000 ml of S-9 mix.

NADP	0.1 M

Check each lot for M.W. as it varies according to impurities present. Filter sterilize and store at -20°C. 200 ml will generate 5000 ml of S-9 mix.

Na_2HPO_4	0.2 M (28.39 g)
distilled water	1 liter

Adjust pH to 7.4 with HCl. Autoclave and store refrigerated. 1 liter will be used in 2000 ml of S-9 mix.

b. Preparation of S-9 Mix (prepare fresh for each test).

For 100 plates, 50 ml of S-9 mix is required. The following is based on 50 ml,

S-9	2-5 ml
KCl + $MgCl_2$ stock	1 ml
Glucose-6-phosphate stock	¼ ml
NADP stock	2 ml
Na_2HPO_4 stock	25 ml

Add sterile distilled water to make 50 ml. It can be stored on ice for several hours.

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Bioassay Systems

Standard Procedure

Section: Genetic Tox.
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Approved: Kenneth S. Loveday

Replaces Original

Subject: Salmonella/microsome Mutagenesis Assay Test Protocol

3. Preparation of Base Layers.

To each liter of bottom agar add:

40% glucose	50 ml
50X VB salts	20 ml

Pour 30 ml per 100 x 15 mm plate. Plates last 2-3 weeks when not refrigerated.

4. Bacterial Tester Strains.

a. Types of bacterial strains:

The five bacterial strains used include:

i. TA 1535, which can be used to detect mutagens causing base pair substitutions.

ii. TA 1537 and TA 1538, which detect frameshift mutagens.

iii. TA 98 and TA 100, which contain a resistance transfer factor (R factor) and are extremely effective in detecting classes of carcinogens which cannot be detected with the other three strains.

b. Storage of strains:

Strains should be stored at -80°C, in a fresh nutrient broth (0.8 ml) with dimethylsulfoxide (0.07 ml) in 2 ml sterile, glass screw-capped vials with rubber-lined screw caps. Each frozen culture is prepared in a duplicate. One is stored as a master copy and is only opened when there is a need to regenerate the "working" stocks. The other is used routinely to obtain fresh cultures for mutagenesis testing by scraping a sterile wooden applicator stick over the surface of the frozen culture (which is not thawed), inoculating nutrient broth (5 ml), and shaking overnight (16 hours maximum) at 37°C. Fresh cultures can be kept in the refrigerator for a week. Tester strains are never subcultured.

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Bioassay Systems

Standard Procedure

Section: Genetic Tox.
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Subject: Salmonella/microsome Mutagenesis Assay Test Protocol

D. METHODS

1. Routine testing of the strains.

For each mutagenesis test, all 5 strains should be tested for the presence of the rfa mutation. This mutation permits large molecules such as crystal violet to enter the bacteria, which in turn inhibit bacterial growth within the crystal violet diffusion zone. At the same time the TA 98 and TA 100 strains are tested for the presence of the ampicillin-resistant R factor. To test the two characteristics, all five strains are streaked in straight lines (use sterile cotton swabs) on a large Mueller-Hinton plate. On one extreme of each streak, sterile filter paper discs containing crystal violet (10 µl of 1 mg/ml) are placed. On the other extreme, ampicillin discs are sterilely placed. There should not be any growth in the crystal violet zone for all five strains. In the ampicillin resistance test, only TA 98 and TA 100 should be seen in the vicinity of ampicillin discs.

Semiannually, all strains should be tested for the uvrB deletion which is very stable and not easily lost from bacteria. To test for the presence of the uvrB deletion, the cultures are streaked on a Mueller-Hinton plate and half of the plate is irradiated for 6 sec. (8 sec. for strains containing the R factor) with a G.E. 15 W. germicidal lamp at a distance of 33 cm. After incubation at 37°C for 12-24 hours, the UV-sensitive strains will grow only on the unirradiated side of the plate. The spontaneous reversion rate of mutation for all five strains should be checked in every test on control plates (the procedure will be described in the section on mutagenesis assays). A back mutation rate which is too high may indicate poor test conditions (for example, top agar temperature too high).

2. Preparation of mutagens and carcinogens.

Solutions of the chemicals are prepared in disposable sterile polystyrene screw-top tubes. The non-water soluble compounds are generally dissolved in DMSO. The bottles of

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DMSO are treated in a sterile manner. Most mutagen solutions can be stored at -20°C for extended periods of time. Solutions of unstable compounds are freshly prepared for each test.

3. Mutagenesis assay.

Place the plates with the base layers upside down, 1/4 open in an incubator for drying out (only if wet, do not overdry). Prepare the S-9 mix, filter it through 0.45 μm millipore filter and store on ice at all times. Melt the top agar and into each 108 ml bottle add 1 ml each of 5 mM biotin and 5 mM L-histidine. Put sterile, capped test tubes in a Dri-bath at a temperature of 49°C and dispense into them 2 ml of molten top agar.

a. Quantitative test:

The following are added (in order) to 2 ml of molten agar: 0.1 ml of the sample to be tested, 0.1 ml of an overnight nutrient broth culture of the bacterial tester strain, and in the case of an activated assay, 0.5 ml of the S-9 mix. Since S-9 is heat labile, it should always be added immediately prior to pouring on the plate.

The contents are mixed by rotating the tube between the palms and poured on the dry bottom agar plate. To accomplish a uniform distribution of the top agar, the plate is tilted and rotated, then set on an even surface to harden. The mixing, pouring, and distribution should take less than 20 sec. and the plates should be left to harden for several minutes. Within an hour the plates should be put in a 37°C incubator. After two days, the arising colonies (representing spontaneous revertants) in both test plates and controls (without the mutagen) are counted.

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Bioassay Systems

Standard Procedure

Section: Genetic Tox.
No.: 080-002
Effective: 10/22/79
Page 7 of 7
Approved: Ernest S. Loveday

Replaces Original

Subject: Salmonella/microsome Mutagenesis Assay Test Protocol

b. Qualitative test:

The mutagen to be tested is omitted from the top agar. Instead it is added (25 µl) to ¼" diameter, sterile, filter discs. The discs are then placed on the hardened top agar, and pressed gently to the surface. The rest of the procedure is exactly the same as for the quantitative test. In both tests the plates are incubated in sealed plastice bags, upside down.

4. General Comments.

a. Each compound and its dilutions should be tested in duplicate on all five strains, both with and without the S-9 mix. Therefore, for each dilution, 20 plates are used: 10 for the activated assay and 10 for the nonactivated assay.

b. Controls, both with and without S-9 mix, are run with each test. It is also advisable to run one known and standardized carcinogen with each test.

c. A compound is considered mutagenic if the arising colonies exceed approximately 2 times the number of spontaneous revertants. The qualitative test is the simplest way for initial screening of several different compounds, or one compound at different dilutions, for mutagenicity or toxicity, respectively.

d. It should be noted that more than one disc can be put in one plate in the qualitative test. The toxicity of a compound is clearly indicated by the size of the zone of inhibition of the background lawn of bacterial growth around the spot. The background lawn does not constitute the colonies. It is a very light bacterial growth due to the addition of some histidine to the top agar. That light growth is the result of growth of a few generations of the bacteria needed for the eventual growth of the colonies. In the case of a positive result, a quantitative test is performed, where steadily increasing dilutions of the same compound are used to construct a dose-response curve.

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APPENDIX D

Laboratory: Biolabs, Inc.
2910 MacArthur Blvd.
Northbrook, IL 60062

Submitter: Dr. Clyde R. Goodheart
President
(312)498-6020

Date of submission: December 5, 1980

Omissions: None

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biolabs

December 5, 1980

Ms. Stephanie Toney
Genetic Bioassay Branch (MD 68)
U.S. EPA
Health Effects Research Laboratory
Research Triangle Park, NC 27711

Dear Ms. Toney:

In accord with your request in your letter of November 26, 1980, enclosed is a copy of our protocols for Ames testing.

I would like to receive a copy of the compilation when it is available.

Yours truly,

CR Goodheart

Clyde R. Goodheart, M.D.
President

CRG/mm

Enclosures

BioLabs Inc. • [illegible] MacArthur Boulevard • Northbrook, Illinois [illegible] • [illegible]

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BioLabs, Inc.

TESTING PROTOCOLS

No. 1003

Page 1 of 4

Approved by Clyde R Goodheart

Effective: 8/25/78

Supersedes: New

Mutagenesis in *Salmonella typhimurium* (without metabolic activation)

Reference: Ames, B.N., McCann, J., and Yamasaki, E. Methods for Detecting Carcinogens and Mutagens with the Salmonella/ Mammalian-Microsome Mutagenicity Test. Mutation Research, 31:349-364, 1975.

1. Introduction

Five mutant tester strains of *S. typhimurium* were selected by Ames, all of which require histidine for growth. They differ in the type of mutation they carry, however. Mutagenic compounds cause the reversion of the mutant to wild-type, able to grow in the absence of histidine; different compounds cause different kinds of back-mutations, so that only one of the tester strains may revert. Three to five (preferably five) dilutions of chemical are used, with triplicate platings. Results are expressed as the number of colonies occurring in the presence of test chemical compared with the spontaneous back-mutation rate in the absence of chemical.

2. Tester strains:

TA-98: R factor introduced into TA1538
TA-100: R factor introduced into TA1535
TA-1575: Strain for detection of base pair substitution
TA-1537: Strain for detection of frameshifts. All strains have uvrB deletion and rfa (deep rough) character.

3. Checking Tester Strains

a. Upon receiving tester strains, inoculate in nutrient broth (8 g Difco-Bacto nutrient broth and 5 g NaCl/liter) and incubate at 32-35°C for 48 hrs.

b. Frozen permanent stocks should be made from broth, after culture is checked for its genetic requirements.

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TESTING PROTOCOLS | Effective: 8/25/78

No. 1003 | Supersedes: New

Page 2 of 4

Approved by

Mutagenesis in *Salmonella typhimurium* (without metabolic activation)

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c. Histidine requirement:

Cultures streaked on minimal plates both with and without histidine (spread 0.1 ml sterile 0.1M ℓ-histidine and 0.1 ml of 0.5mM biotin on the agar surface).

d. Test for deep rough (rfa) character by testing for crystal violet sensitivity.

After 12 hours incubation at 37°C of a 10^8 bacteria culture, a sterile paper disc containing 10 μl of 1 mg/ml crystal violet solution should produce a clear zone around the disc (approx. 14 mm in dia.) on nutrient agar.

e. New tester strains with R factors (TA100 & TA98) should be checked routinely for the presence of the ampicillin resistant R factor.

Culture cross streaked against a dry streak of 10 μl of 8 mg/ml in 0.02 N NaOH of ampicillin on surface of nutrient agar and incubated at 37°C for 12-24 hours. Strains not containing R factors will show zone of inhibition.

f. Checking for uvr B deletion

Check UV sensitivity of strains by Cross-streaking culture on nutrient agar and irradiating half of the streak for 6 sec. (8 sec. for R factor strains) with a G.E. 15 watt germicidal lamp at a distance of 33 cm. After incubation at 37°C for 12-24 hours, the UV-sensitive strains will grow only on unirradiated side.

g. Spontaneous reversion rate sould be checked. Abnormally high rate may indicate contamination of culture.

4. Storage:

Storage of R factor and standard tester strains is at -80°C in 0.8 ml of fresh nutrient broth with 0.07 ml DMSO in sterile glass screw top vials.

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Approved by Clyde R Goodheart

Mutagenesis in *Salmonella typhimurium* (without metabolic activation)

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5. Test chemical concentrations:

No less than 3 concentrations in an appropriate solvent in 1/2 log increments are tested initially. Should the high concentrations appear to be cidal, permitting no growth of any or all tester strains, the array of concentrations is adjusted downward. Final test concentrations bracket the effective/no effect parameters of the first assays.

6. Preparation of mutagens:

   a. Solutions prepared in sterile polystyrene screw cap tubes.

   b. DMSO - dimethylsulfoxide used for compounds which are not water soluble - max of 0.5 ml per plate.

   c. p-Dioxane or ethanol can also be used as solvents up to 100 µl of either solvent per plate.

   d. Most mutagenic solutions can be stored at -20°C.

   e. Highly reactive compounds should be freshly prepared.

7. Mutagenesis assays on plates

Media:

Top agar: 0.6% Difco Agar
0.5% NaCl

Autoclave in 100 ml volumes and keep at room temperature before use:

Agar melted in steam and 10 ml of a sterile solution of 0.5mM ℓ-histidine. HCl and 0.5mM biotin is added to the molten top agar and mixed thoroughly by gentle swirling.

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No. 1003

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Approved by Clyde R Goodheart

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Supersedes: New

Mutagenesis in *Salmonella typhimurium* (without metabolic activation)

Minimal-glucose agar medium	1.5% Difco Agar 2.0% Glucose

in Vogel-Bonner Medium E

8. Determine counts of plates containing the array of chemical concentrations assayed as well as those of a positive control, solvent control, and spontaneous revertants. Subtract counts for spontaneous revertants obtained for each test strain from the counts obtained for all other assays made with each strain.

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TESTING PROTOCOLS — Effective: 8/25/78

No. 1004 — Supersedes: New

Page 1 of 2

Approved by Clyde R Goodheart

Induction and Preparation of Rat Liver Enzymes and S-9 Mix

Reference: Ames, B.N., McCann, J., and Yamasaki, E. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Research, 31:349-364, 1975.

Induction of Rat Liver Enzymes

a. Preparation of animals

1. Male rats - 200-300 g each (Charles River, Wistar strain, Charles River Breeding Lab., Inc.)
2. Single injection of Aroclor 1242 or test chemical to be used as activant (diluted in corn oil to a conc. of 200 mg/ml) I.P. at a rate of 500 mg/kg five days before sacrifice.
3. Drinking water given *ad libitum* and Purina Laboratory Chow until 12 hours before sacrifice.
4. On fifth day of induction, rats are killed by cervical separation, decapitated and bled.

b. Preparation of liver homogenate fraction (S-9)

1. All steps are at 0-4°C using cold, sterile solutions and glassware.
2. Livers placed in beakers containing 0.15M KCl (approx. 1 ml/g of wet liver) for weighing.
3. After weighing, livers are transferred to a beaker containing 0.15M KCl (3 ml/g wet liver) and minced with sterile scissors, and homogenized in a Potter-Elvejham apparatus with a Teflon pestle.
4. Centrifuge the homogenate for 10 min at 9000 × g (8700 rpm).

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TESTING PROTOCOLS — Effective: 8/25/78

No. 1004 — Supersedes: New

Page 2 of 2

Approved by Clyde R Goodheart

Induction and Preparation of Rat Liver Enzymes and S-9 Mix

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5. Decant the supernatant and save (S-9 fraction).
6. Fresh liver homogenated and centrifuged fractions are distributed in 2 ml portions.
7. Quickly freeze and store at -80C.

c. Preparation of S-9 mix

S-9 mix contains per ml: S-9 (0.04-0.1 ml) used 50 μl/ml
$MgCl_2$ 8μM
KCL 33 μM
Glucose -6-phosphate 5μM
NADP 4μM
Sodium phosphate Buffer pH 7.4 100μM

S-9 mix is prepared freshly each day, filter sterilized, and kept on ice before and during use.

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BioLabs, Inc.

TESTING PROTOCOLS

No. 1005

Page 1 of 2

Approved by: Clyde B Goodheart

Effective: 8/25/78

Supersedes: New

Mutagenesis in *Salmonella typhimurium* (with metabolic activation)

Reference: Protocol 1003 and 1004

All procedures conform to those of Protocol 1003 except as indicated hereunder.

2. Test strains

    add: TA-1538

7. Mutagenesis assays on plates

    a. Media:

        Top Agar: 0.6% Difco Agar
        0.5% NaCl

Autoclaved in 100 ml volumes and kept at room temperature before use.

Agar melted in steam and 10 ml of a sterile solution of 0.5μM ℓ-histidine·HCl and 0.5μM biotin is added to the molten top agar and mixed thoroughly by gentle swirling.

Minimal-glucose agar medium in Vogel-Bonner Medium E	1.5% Difco Agar 2.0% Glucose

    b. Mutagenesis assay procedure with S-9 mix (see Protocol 1004).

        1. Added in order to 2 ml molten top agar at 45°C:
            a. 0.1ml of overnight nutrient broth culture of bacterial tester strain.
            b. Sample to be tested.
            c. 0.5 ml of the S-9 mix.
            d. S-9 mix should not be left at 45°C for more than a few seconds.

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Page 2 of 2

Approved by: Clyde R Goodheart

Effective: 8/25/78

Supersedes: New

Mutagenesis in *Salmonella typhimurium* (with metabolic activation)

2.a. The contents are mixed by rotating the tube between the palms.

b. The contents of the tube are then poured on minimal glucose agar plates.

c. Uniform distribution of the top agar is accomplished by gently tilting and rotating the uncovered plate, and then setting down to harden.

d. Mixing, pouring, and distributing should take less than 20 seconds.

8. Control Reagents

Positive control: 2-aminofluorene, Aldrich Chemical Company
Solvent: Dimethylsulfoxide, Mallinkrodt, if test material is not water soluble.

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APPENDIX E

Laboratory: Bio-Technics Laboratories, Inc.
1133 Crenshaw Blvd.
Los Angeles, CA 90019

Submitter: Dr. Rae E. Drazin
Chairman, Biology/Microbiology
(213)933-5991

Date of submission: December 4, 1980

Omissions: None

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BIO-TECHNICS LABORATORIES, INC.

December 4, 1980

Stephanie Toney
USEPA, HERL, GTD, GBB (MD-68)
Research Triangle Park, NC 27711

Dear Ms. Toney:

As you requested in your letter of November 26, we are pleased to submit a copy of our current Ames Test protocol. A compilation of Ames test protocols is a very worthwhile undertaking, for the reasons you stated, and we would appreciate a copy of the compilation when completed.

It is the policy of Bio-Technics Laboratories to furnish protocols with the understanding that they be viewed as company confidential documents. We trust you will honor their proprietary nature.

Please correct your files to note that the director of Bio-Technics Laboratories is R.J. Schlesinger, Ph.D. Mr. Matthew J. Steiger was Manager of Customer Relations.

Cordially,

Rae E. Drazin, Ph.D.
Chairman, Biology/Microbiology

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Encl.

BIOTECH
1133 Crenshaw Boulevard · Los Angeles, California 90019
Phone 933-5991 – Cable Address: BITEC
TWX 910-321-2862

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BIO-TECHNICS LABORATORIES, INC.

January 20, 1981

Ms. Stephanie H. Toney
USEPA, HERL, GTD, GBB (MD-68)
Research Triangle Park, North Carolina 27711

Dear Ms. Toney,

In reply to your letter of January 13, we herewith withdraw our confidentiality restrictions and authorize the EPA to utilize our Ames Test Protocol in your laboratory protocol compilation.

We look forward to a final copy of the compilation.

Cordially,

Rae E. Drazin

Rae E. Drazin, Ph.D.,
Chairman, Microbiology/Biology

RED:ms

1133 Crenshaw Boulevard · Los Angeles, California 90019
Phone 933 5991 — Cable Address: BITEC
TWX 910-321-2862

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BIO TECHNICS LABORATORIES
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2-2-131-79
3-2-079-79

6/13/80

AMES TEST (QUANTITATIVE)
(BTP 3-2-079-79 & BTP 2-2-131-79)

Introduction: The Ames Test previously described (BTP 2-2-099-78) was essentially a qualitative test, thus, a modified procedure was devised in order that quantitative results can be obtained. The main difference between the two (2) procedures will be in the manner in which the test sample is introduced to the agar plate. Steps 1-8 described in BTP 2-2-099-78 are identical in the qualitative and quantitative assay.

Reference: Modified from Ames, Bruce N., et al., Mutation Research, 51, 1975 (pp. 347-364); and Yahagi, et al., Cancer Letters, 1, 1975, pp. 91-96.

1.0 Principle In the presence of mutagenic (and potentially carcinogenic) substances, histidine auxotrophic stains of *Salmonella typhimurium* often revert to the histidine prototrophic wild type. This reversion is measured both with and without activation by a rat-liver microsome fraction (S-9). The test material (a solid, crystal or microdrop of a liquid) is placed on the surface of an agar plate containing tester micro-organisms. If the substance is mutagenic, revertant colonies will appear as a "halo" in a zone around the sample. This is a screening procedure which yields essentially qualitative rather than quantitative results.

1.1 All technicians performing this test are required to read thoroughly the reference paper listed in this protocol before beginning the assay.

2.1 Equipment:
- 2.1.1 37°C incubator
- 2.1.2 Shaker for aerating cultures
- 2.1.3 - 20°C Freezer
- 2.1.4 0 - 4°C Refrigerator
- 2.1.5 100 x 15 mm sterile plastic dishes
- 2.1.6 *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538
- 2.1.7 Water bath set at 45°C

2.2 Reagents
- 2.2.1 NaCl
- 2.2.2 Nutrient Broth (Difco or BBL)
- 2.2.3 0.0001 M l-Histidine sterilized by membrane filtration and stored at 0 - 4°C
- 2.2.4 0.0005 M. Biotin sterilized by membrane filtration and stored at 0 - 4°C
- 2.2.5 1 mg/ml crystal violet
- 2.2.6 2 mg/ml deoxycholate
- 2.2.7 0.8 mg/ml Ampicillin (in 0.2 N NaOH)

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BTL PROCEDURE NO 2-2-131-79 3-2-079-79
Written by ______ Appr'd by ______ Date
Revised by J Appr'd by Dwyer Date 6/13/

2.2.8	Aroclor 1254 (Analabs)
2.2.9	0.4 M $MgCl_2$, sterilized by autoclaving
2.2.10	0.65 M KCl , sterilized by autoclaving
2.2.11	0.2 M $NaPO_4$, pH 7.4; sterilized by autoclaving
2.2.12	0.1M NADP , sterilized by membrane filtration and stored at - 20°C.
2.2.13	1.0 m Glucose-6-phosphate, sterilized by membrane filtration and stored at - 20°C.
2.2.14	Top Agar: Top agar (0.6% Difco agar, 0.5% NaCl) in autoclaved and stored in bottles in volumes of 100 ml at room temperature. Before use, the agar is melted by heating the bottles in a steam bath. 10 ml of a sterile solution of 0.5 mM l-histidine-HCl-0.5 mM biotin is added to the molten top agar and mixed thoroughly by gentle swirling.
2.2.15	S-9 mix, prepared as described below (Section 7.0).
2.2.16	Dimethyl Sulfoxide (DMSO)
2.2.17	Methyl Methanesulfonate (MMS)
2.2.18	N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (0.4 mg/ml in DMSO)
2.2.19	9-aminoacridine (2 mg/ml in ethanol)
2.2.20	2-aminofluorene (2 mg/ml in DMSO)
2.2.21	Aflotoxin B_1 (0.2 mg/ml in DMSO)
2.2.22	Davis Minimal Agar (Difco)

3.0 Genotype of the Strains Used for Mutagen Testing

Histidine mutation			Additional mutations			Use
hisG46	hisC3076	hisD3052	LPS	Repair	R factor	
TA1535 TA100	TA1537	TA1538 TA98	rfa rfa	ΔuvrB ΔuvrB	- +R	Standard tester strains and R factor strains; recommended for general mutagenesis screening

3.1	hisG46 is a base pair substitution mutation
3.2	hisC3076 is a frame-shift mutation involving an alternating sequence of cytosine and guanine residues.
3.4	LPS refers to the Lipopolysaccharide bacterial cell envelope, and rfa is a mutation which results in a rough cell character.
3.5	ΔuvrB is a deletion mutant of the nitrate reductase (chl) biotin (bio) and uvrB genes. This gene controls the repair of certain lesions in DNA.
3.6	The R factor is pKM_{101} .

4.0 Storage and general handling of tester strains

4.1	For permanent storage of tester cultures, see BTP 2-2-118-78.
4.2	Working cultures used as the inoculum for the actual tests are stored at 0 - 4°C in the refrigerator on Petri plates. (see below, 4.3).

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4.3 Prepare Davis minimal agar plates
- 4.3.1 Pipette 0.1 ml of sterile 0.1M L-histidine onto the surface and distribute evenly with a sterile glass spreader.
- 4.3.2 Add 0.1 ml of sterile 0.5 mM biotin as in 4.3.1.
- 4.3.3 For the R-factor strains (TA98 and TA100), add 0.1 ml of a sterile 8 mg/ml solution of Ampicillin (in 0.02N NaOH). Deliver Ampicillin evenly over the plate so as to avoid concentration in any one area.

4.4 When recovering R-factor containing strains from permanent storage, add Ampicillin at 25 μg/ml to the nutrient broth in order to maintain selective pressure for the plasmid.

4.5 Preparation of Master Plates
- 4.5.1 Inoculate nutrient broth (with or without ampicillin, as required; see above) from the permanent storage culture. Incubate at 37°C for 15 - 16 hours with vigorous aeration. TA1537 and TA1538 are somewhat temperature sensitive, so the temperature must not exceed 37°C.
- 4.5.2 Streak some of the nutrient broth cultures onto the surface of Davis minimal glucose agar plates. Include ampicillin in the plates used for the R-factor containing strains.
- 4.5.3 Incubate at 37°C for 24 hours.
- 4.5.4 Store these Master Plates at 0 - 4°C. They can be used for several weeks to several months.
- 4.5.5 Immediately after preparation of the master plates, confirm the genetic/physiologic characters of the tester strains as described in section 5 below.

4.6 Re-Isolation of Tester Stains from Permanent Stock Cultures. Occasionally, the tested strains will be mixtures of different genetic variants of the original strain. The parental strain can often be reisolated from the mixture in the following way.
- 4.6.1 Inoculate nutrient broth cultures (with or without ampicillin, as required).
- 4.6.2 Incubate at 37°C for 16 - 20 hours.
- 4.6.3 Streak on Davis minimal glucose plates (prepared as described above).
- 4.6.4 Incubate at 37°C for 24 - 36 hours.
- 4.6.5 Pick cells from at least 5 colonies, grow each in nutrient tubes (with ampicillin added for the plasmid containing strains).
- 4.6.6 Prepare new master plates from each isolate.
- 4.6.7 Check the genetic/physiologic character of each isolate.
- 4.6.8 Place into permanent storage the isolate which corresponds to the parental strain.

4.7 Replacement of Master Plates
- 4.7.1 When the master plates begin to dry out, make new ones as described in 4.5.
- 4.7.2 Confirm the genetic and physiologic character of organisms on these new plates as describ ed in section 5 below.
- 4.7.3 Do not make new master plates from old master plates. Use only the permanent stock cultures.

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4.8 Storage of Liquid Cultures

4.8.1 Whenever possible, fresh nutrient broth cultures should be used for mutagenesis testing (no ampicillin added in any case).

4.8.2 These nutrient broth cultures may be stored at 4°C for 1 - 2 days, but must not be used after 2 days.

5.0 Testing of Cultures for their Genetic/Physiologic Character

5.1 Histidine Requirement

5.1.1 Streak the cultures on minimal agar plates with and without histidine added.

5.1.2 For strains containing the uvrB deletion, also add biotin to the plates.

5.1.3 It is convenient, and a saving of plates, to divide a single plate into several sectors and inoculate a single sector with each organism.

5.1.4 Incubate at 37°C for 24 - 36 hours.

5.1.5 All strains should grow in the presence of histidine, and none should grow in its absence.

rfa Character - All Strains

5.2.1 Stains having the deep rough (rfa) character are tested for their crystal violet sensitivity.

5.2.2 Saturate a sterile filter paper disc (1 cm diameter) with an aqueous solution of 1 mg/ml crystal violet.

5.2.3 Pipette 0.1 ml (about 10^8 cells) of an overnight nutrient broth culture of the test organism into 5 ml of molten (45°C) Davis minimal agar.

5.2.4 Pour the agar onto the surface of a previously prepared nutrient agar plate.

5.2.5 After the agar has hardened, place the disc containing crystal violet on the surface.

5.2.6 Incubate for 12 - 16 hours at 37°C.

5.2.7 A clear zone of inhibition of all growth around the disc indicates the presence of the rfa mutation.

5.3 R-factor Character

All strains, but most especially TA98 and TA100 should be checked routinely before each use for the presence (or absence) of the R-factor.

5.3.1 Streak a small amount (10µl) of an 8 mg/ml solution of ampicillin across a 100 mm petri dish. Use care in not allowing the solution to concentrate in any one area. Allow the solution to dry.

5.3.2 Cross streak the cultures to be tested at 90° to the ampicillin streak.

5.3.3 Incubate at 37°C for 16 - 24 hours.

5.3.4 Strains which do not contain the F factor will be inhibited in their growth in a zone around the ampicillin streak. R-factor containing strains will grow normally.

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5.4 uvrB Deletion.

Perform this test on all stains.

5.4.1 Cross streak cultures on the surface of a nutrient broth plate (supplemented with histidine and biotin).

5.4.2 Cover half of the plate with a u.v. opaque shield (black paper, a piece of glass).

5.4.3 Irradiate the plate for 6 - 8 seconds with a germicidal light (G.E. 15 watt) held at a distance of 33 cm.

5.4.4 Incubate the plates at 37°C for 16 - 24 hours.

5.4.5 The u.v. sensitive strains should not grow on the irradiated section of the plate.

6.0 Induction of rat liver enzymes for mutagen activation.

6.1 Dilute Aroclar 1254 to a concentration of 200 mg/ml in corn or sesame oil.

6.2 Inject male rats at a dose of 500 mg/kg of body weight.

6.3 The rats are given water ad libitum and a normal feeding of standard Laboratory Chow (Purina or the equivalent).

6.4 Remove food and water 12 hours before sacrifice.

6.5 On the fifth day after injection, sacrifice the rats and remove the liver.

7.0 Preparation of Liver Homogenate Fraction (S-9).

7.1 All steps are at 0 - 4°C using cold, sterile solutions and glassware.

.2 The livers (rat livers are about 10 - 15 g each) are placed in beakers (pre-weighed) containing sterile 0.15 M KCl (approximately 1 ml/g wet liver).

7.3 After weighing, livers are transferred to a beaker containing 3 volumes of 0.15 M KCl (3 ml/g wet liver), minced with sterile scissors, and homogenized in a Potter-Elvehjem apparatus with a Teflon pestle.

7.4 The homogenate is centrifuged for 10 min at 9000 g (8700 rev./min in SS -34 head of Sorvall RC_2-B) and the supernatant, which we call the S-9 fraction, is decanted and saved.

7.5 One ml of S-9 fraction contains microsomes from about 250 mg of wet liver; protein concentration is about 40 mg/ml.

7.6 The fresh S-9 fractions are distributed in 2-ml portions in small sterile plastic tubes, quickly frozen in dry ice, and stored at -80° in a Revco freezer.

7.7 As required, sufficient S-9 fraction is thawed (at room temperature) and kept on ice; the unused portion is discarded at the end of the day.

7.8 The extent of bacterial contamination of the S-9 should be determined; if necessary the S-9 mix may be filter sterilized.

.0 Preparation of S-9 mix.

8.1 S-9 mix contains per ml: S-9 (0.04 - 0.1 ml), $MgCl_2$ (8 micro moles), KCl (33 micro moles), Glucose-6-phosphate, (5 micro moles), NADP (4 micro moles), and sodium phosphate, pH 7.4 (100 micro moles).

8.2 Stock solutions of NADP (0.1 M) and glucose-6-phosphate (1M) are prepared with sterile water in sterile tubes and stored at -20°C.

8.3 The stock salt solution 0.4 M $MgCl_2$, 0.65M KCl, and phosphate buffer, 0.2M, pH 7.4) are autoclaved and stored in the refrigerator.

8.4 S-9 mix is freshly prepared each day and may be kept on ice several hours before use.

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Ames Test (Quantitative)
BTP-3-2-079-79 &
BTP-2-2-131-79
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8.5 Prepare the S-9 Mix as follows:

Stock	**Volume/ml**
S-9	0.04 - 0.1 ml
0.4 M $MgCl_2$	20 µl
0.65 M KCl	30 µl
0.2 M Na_3PO_4, pH 7.4	0.5 ml
0.1 M NADP	40 µl
1 M Glucose-6-PO_4	5 µl
Sterile Deionized Water	to 1 ml

9.0 Assay Procedure (Modified)

9.1 The following are preincubated at 37°C for twenty (20) minutes: 0.1 ml of an overnight nutrient broth culture of a tester strain, 0.1 ml of the appropriate concentration of the test sample and 0.5 ml of the S-9 mix (if required), after which 2.0 ml of soft agar is added and the mixture poured over the minimal agar plates. This is an initial screening procedure; therefore, a wide concentration range is used. Concentrations of 0.2, 2, 20, 50 and 500 ug of samples per plate may be used both in the absence and presence of the standard S-9 mix. (Note: For azo dyes five (5) concentrations from 0.1 to 1 micromole are appropriate.)

9.2 The contents are mixed (by rotating the tube between the palms) and poured on minimal glucose agar plates. Uniform distribution of the top agar on the surface of the plates is accomplished by gently tilting and rotating the uncovered plate and then setting the plates down to harden.

9.3 The mixing, pouring and distribution should take less than twenty (20) seconds and the plates should be left to harden several minutes. It is important to follow these time limits. If the top agar starts to harden in mid-operation, a stippled plate surface will result which makes scoring of revertants difficult.

9.4 As soon as possible, and certainly within an hour, the plates should be put in a dark, 37°C incubator.

9.5 After two (2) days, the colonies (revertants to histidine prototrophy) in both test plates and controls are counted, and the presence of the light background lawn of growth (due to the trace amounts of histidine added) is confirmed.

9.6 Sponteneous revertant colonies on control plates without mutagen and S-9 mix are about (at 48 hours): 20 (TA1535), 7 (TA1537), 25 (TA1533), 160 (TA100), and 40 (TA98). Slightly larger number of spontaneous revertant colonies arise on plates containing the S-9 mix.

9.7 In addition to the test of the sample, positive and negative controls are included in the test.

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9.7.1 Negative Control.
A 10 ul drop of sterile saline is applied to the surface of the appropriate petri dish.

9.7.2 Positive Control.
Chemical mutagens are used, for each type of tester strain. Their identity and concentration are:

9.7.2.1 Methyl metnane-sulfonate (MMS).
This mutagen is effective on strain TA100. Two (2) microliters of the undiluted liquid are spotted on the surface of the appropriate tester plates (see below).

9.7.2.2 N-methyl-N^1-Nitro-N-Nitrosoguanidine (MNNG).
This mutagen is effective on strains TA1535 and TA100. Five (5) microliters of a PO.4 mg/ml Dimethylsulfoxide (DMSO) solution are spotted on the surface of the appropriate tester plates (see below).

9.7.2.3 9 - amino acridine (9-AA).
This mutagen is effective on strain TA1537. Five (5) microliters of a 2 mg/ml ethanol solution are spotted on the surface of the appropriate tester plates (see below).

9.7.2.4 2 - Aminofluorene (2-AF).
This mutagen is effective on strains TA100, TA98 and TA1535 only when activated by the microsome fraction. Five (5) microliters of a 2 mg/ml DMSO solution are spotted on the surface of the appropriate tester plates (see below).

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9.8 Summary Table of the Test Set-Up.

Specimen		[1]Metabolic Activation	TA 98	TA 100	TA 1535	TA 1537	TA 1538
Negative Control		+	5 µl	5 µl	5 µl	5 µl	5 µl
		−	5 µl	5 µl	5 µl	5 µl	5 µl
[2]Positive Controls	a) MMS	+	2 µl	2 µl	2 µl	2 µl	2 µl
		−	2 µl	2 µl	2 µl	2 µl	2 µl
	b) MNNG	+	5 µl	5 µl	5 µl	5 µl	5 µl
		−	5 µl	5 µl	5 µl	5 µl	5 µl
	c) 9-AA	+	5 µl	5 µl	5 µl	5 µl	5 µl
		−	5 µl	5 µl	5 µl	5 µl	5 µl
	d) 2-AF	+	5 µl	5 µl	5 µl	5 µl	5 µl
		−	5 µl	5 µl	5 µl	5 µl	5 µl
	e) AFB_1	+	5 µl	5 µl	5 µl	5 µl	5 µl
		−	5 µl	5 µl	5 µl	5 µl	5 µl
Test Sample Initial Screen	1	±					
	2	±					
	3	±					
	4	±					
	5	±					
Test Sample Lower dose ranges	1	±					
	2	±					
	etc.						

(Tester Strain: TA 98, TA 100, TA 1535, TA 1537, TA 1538)

[1] Sample incubated with (+) or without (−) rat liver microsome fraction (S-9)
[2] Positive Controls: MMS = Methyl Methanesulfonate
MNNG = N-methyl-N'-Nitro-N-Nitrosoguanidine (0.4 mg/ml)
9-AA = 9-aminoacridine (2 mg/ml)
2-AF = 2-aminofluorene (2 mg/ml)

9.9 Repeat assay procedure (9.1-9.7) at a narrower dose range if there was a positive result at any concentration used during the screening procedure for any of the tester strains. The test is considered positive if the number of revertant colonies on the test sample plates is a factor of 2 or more greater than the number on the negative control plates (spontaneous revertants). The lowest concentration at which the test was positive should be used as an upper end-limit for the dose-response assay (i.e. if the test sample was positive at 0.2 ug, a range between 0.1 ug and 0.2 ug can be conducted).

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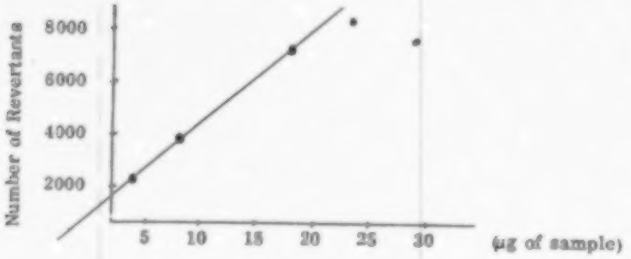
10.0 Data Collection and Interpretation

10.1 Before control plates and test plates are observed for the presence of revertant colonies, they should routinely be examined to check the condition of the background lawn. If massive cell death has occurred, the background lawn on the test plates will be sparse compared to the control plates.

10.2 Record the number of revertant colonies for each test strain on both the control and the sample plates. Also record the concentrations of the test materials, along with the corresponding number of revertants.

10.3 The number of revertants observed is placed on the vertical axis and the concentration of test sample used will be placed on the horizontal axis. If 3 points within the range of concentrations are linear, the test is completed and results recorded.

For this quantitative assay, we are interested in the effect of the concentration of the suspected mutagenic sample on the number of revertants. In general, for most mutagens, a concentration range exists which yields a linear dose-response. It is in this range that the number of revertants per plate reported for any mutagen should be taken. In some cases, the graph will not be linear at higher concentrations since the mutagen may be highly toxic and, therefore, may cause excessive bacterial killing. This causes a decrease in the number of revertants on the plate. An example of a completed graph is constructed below.



If the completed graph shows a linear response, the mutagenic activity per unit (concentration) of test sample can be calculated. Select any point along the linear segment of the graph and note the number of revertants and the concentration of test sample used. For the particular graph (see above), the mutagenic activity will be 2500 revertants per 5 µg or after simplification 500 revertants per µg of test sample. It is important that a point be taken from the linear range, otherwise, the calculated activity of the sample will not be reliable and reproducible.

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APPENDIX F

Laboratory: Case Western Reserve University
Department of Pediatrics
School of Medicine
2103 Adelbert Rd.
Cleveland, OH 44106

Submitter: LeRoy Meyer

Date of submission: February 12, 1981

Omissions: None

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CASE WESTERN RESERVE UNIVERSITY · CLEVELAND, OHIO 44106

February 12, 1981

Stephanie Toney
USEPA, HERL, GTD, GBB (MD-68)
Research Triangle Park, NC 27711

6-1/2"

Dear Ms. Toney:

In answer to your letter of January 16, 1981, please find enclosed a copy of our Ames Test Protocol. An earlier protocol was sent to you in December but apparently was lost in the mails. Should there be any questions concerning the protocol, please do not hesitate to contact us.

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Sincerely yours,

LeRoy Meyer

Enclosure

acv

Department of Pediatrics
School of Medicine
Rainbow Babies and Childrens Hospital

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AMES TEST PROTOCOL

1. 5ml of Columbia broth is inoculated with a loop of culture taken from a slant refrigerated at 4°C. This culture is allowed to incubate overnight.
2. A 1:20 dilution is made from the overnight and is allowed to incubate until the concentration is greater than 1×10^8 organisms per ml. Generally, three to four hours is required, depending on the strain. Time for growth has been determined both by means of growth curves and a measure of absorbance at 450 nm (0.8 absorbance indicates cells in excess of 1×10^8).
3. Compounds which are not water soluble are generally dissolved in dimethylsulfoxide (DMSO). Sample size ranges between 10 ul and 100 ul. Solvent controls receive same volume as test samples.
4. Preparation of "S-9" fraction is as detailed in Ames et al. (1975). Both hamster and rat are used.
5. 0.1 ml of 1:20 dilution culture, 10 ul - 100 ul of test, positive or solvent control chemical and 0.5 ml of either buffer (pH=7.4) or S-9 mix are added to test tube and preincubated 20 minutes at 37°C. After 20 minutes, 2 ml of agar overlay is added and mixed, then poured on Davis medium with Biotin.
6. The plates are incubated at 37°C for 48 hours.
7. The numbers of revertants/plate are counted.

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MEDIA FOR AMES ASSAY

1. Columbia Broth - BBL

   Columbia broth - 35 g + 1 liter H_2O

   Dissolve, distribute 100 ml per 250 ml flask.
   Plug with cotton + gauze and autoclave 30 minutes.

2. DMB Agar Plates (Davis medium with biotin)

   a. 3.0 g KH_2PO_4

      7.0 g $K_2H\ PO_4$

      1.0 g $(NH_4)_2\ SO_4$

      0.5 g Sodium citrate

   Dissolve in 500 ml H_2O (in 1 liter flask)

   Add 0.4 ml of 1M $MgSO_4$

   b. 15.0 g granulated agar
      5.0 g Dextrose

   Dissolve in 500 ml H_2O (in 1 liter flask).

   Autoclave 2a + 2b for 30 minutes

   Mix and pour into petri dishes.

3. DMH Soft Agar (Davis medium with histidine)

   a. 0.6 g KH_2PO_4

      1.4 g $K_2H\ PO_4$

      0.2 g $(NH_4)_2\ SO_4$

      0.1 g Sodium citrate

   Dissolve in 100 ml H_2O (in 250 ml flask)

   add 0.08 ml 1 M $MgSO_4$
   add 0.04 ml Histidine, 0.1 mg/ml

   b. 1.2 g granulated agar
      1.0 g Dextrose

   Dissolve in 100 ml H_2O (in 250 ml flask)

   Autoclave 3a + 3b for 30 minutes and mix.

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APPENDIX G

Laboratory: EG & G Mason Research Institute
1530 E. Jefferson St.
Rockville, MD 20852

Submitter: Dr. Steve R. Haworth
Director, Genetic Toxicology
(301)770-4400

Date of
submission: December 31, 1980

Omissions: None

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EG&G MASON RESEARCH INSTITUTE
1530 EAST JEFFERSON STREET, ROCKVILLE, MARYLAND 20852 • TEL. (301) 770-4400

December 31, 1980

Ms. Stephanie Toney
Genetic Bioassay Branch
USEPA, HERL, GTD, GBB (MD 68)
Research Triangle Park
North Carolina 27711

Dear Ms. Toney:

As per your request, I am enclosing a copy of our standard protocol for conducting the Salmonella/mammalian-microsome mutagenicity assay (Ames Test).

I would appreciate receiving a final copy of the compilation of Ames test protocols.

If you have any questions concerning our protocol, please feel free to contact me.

Sincerely,

Steve R. Haworth

Steve R. Haworth, Ph.D.
Director
Genetic Toxicology

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1530 EAST JEFFERSON STREET, ROCKVILLE, MARYLAND 20852 • TEL. (301) 770-4400

Protocol 1.0 7/80

Salmonella/Mammalian-Microsome Plate Incorporation Mutagenicity Assay (Ames Test)

Sponsor:

Testing Facility: EG&G Mason Research Institute
1530 East Jefferson Street
Rockville, Maryland 20852

Purpose: To evaluate the mutagenic potential of the test article using the Ames Test.

Reason for Choice: The Ames Test has been shown to be a sensitive, rapid, accurate indicator of the mutagenic activity of a broad range of chemical classes.

Route of Administration: Plate incorporation both with and without metabolic activation.

Reason for Choice: This approach has been shown to be capable of detecting a wide range of mutagens.

Test Article I.D.*:

*This test article has been adequately characterized by the sponsor.

Proposed Starting Date:

Proposed Completion Date:

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The stipulations of this protocol will be implemented in conformance with Good Laboratory Practice Regulations (21 CFR, Part 58) for nonclinical laboratory studies.

Will this study be submitted to a regulatory agency?_____
If so, to which agency or agencies?______________________

____
Sponsor's Study Coordinator

____
Date Approved by Sponsor

____
Study Director Date

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EXPERIMENTAL PROCEDURES

Tester Strains

The tester strains to be used will be the histidine auxotrophs TA98, TA100, TA1535, TA1537 and TA1538, which were obtained directly from Dr. B. Ames and have since been stored in liquid nitrogen.

Media

Top agar will be initially prepared with 8 g/liter Difco Bacto Agar and 5 g/liter NaCl. After autoclaving, the molten agar will be distributed in 100 ml aliquots into sterile bottles where it will be stored at room temperature. Immediately before its use in the mutagenesis assay, the top agar will be melted and supplemented with 10 ml/100 ml agar of a sterile solution containing 0.5mM L-histidine and 0.5mM biotin (1XSA). Twenty-five ml of sterile deionized water will be added per 100 ml top agar when it is used in assays without metabolic activation. This insures that final top agar and amino acid supplement concentrations will be the same on plates with or without metabolic activation.

10XSA supplement will contain 5.0mM L-histidine and 0.5mM biotin.

Bottom agar will be Vogel-Bonner minimal medium E described by Ames.

Nutrient broth used for growing overnight cultures of the tester strains will contain 25g per liter of Nutrient Broth No. 2 (Oxoid).

Standard solvents used for diluting test articles are, in order of preference, deionized, distilled H_2O, dimethylsulfoxide, acetone and ethanol. Other solvents can be used upon request.

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Storage and Preparation of Tester Strains

All tester strains are stored in liquid nitrogen, and fresh cultures will be inoculated directly from these frozen stocks. Broth cultures will be grown overnight at 37°C with shaking. If necessary, the cultures will then be centrifuged and resuspended in their culture medium to give an appropriate final cell concentration. Each culture will be checked for crystal violet sensitivity and ampicillin resistance at the time of its use in the mutagenesis assay.

Labelling of Experimental Procedures

Agar Batch Number

Vogel Bonner minimal bottom agar will be routinely prepared in 5 liter batches and dispensed in 25 ml aliquots into 15 x 100 mm sterile Falcon plastic Muta-Assay petri dishes. Each agar batch consisting of approximately 200 plates will be given a unique sequential number, and the lid of each plate containing agar from that batch will be stamped in permanent ink with that number. The lot number of the plastic petri dishes used will also be recorded. Documentation of this procedure will be recorded in the appropriate section of the media notebook.

Plate Labelling Procedures for Preliminary Toxicity Experiment

A felt tipped pen containing permanent ink will be used to label all plates. All markings will be placed on the side of the bottom of the petri dish.

Each experiment will be identified with the MRI# and the capital letter "A". "A" will always designate that the plate will be used in a preliminary toxicity study. The first preliminary toxicity study on a given test article will be identified as A1. If the experiment needs to be repeated, the repeat experiment will be identified as A2. If still another repeat is required, it will be identified as A3. The plates

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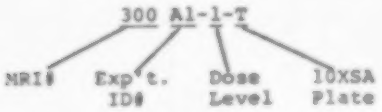
containing undiluted cells and 1XSA top agar will be identified by the letter M. The plates containing diluted cells and 10XSA top agar will be identified by the letter T. The dose levels will be identified by the following code numbers:

0 - Solvent
8 - Eighth test article dilution
7 - Seventh test article dilution
6 - Sixth test article dilution
5 - Fifth test article dilution
4 - Fourth test article dilution
3 - Third test article dilution
2 - Second test article dilution
1 - First test article dilution



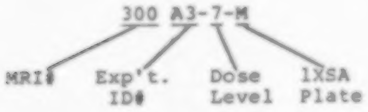
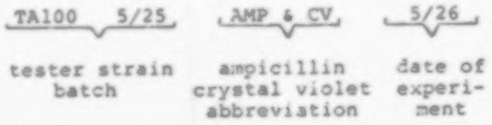


Plate Labelling for Tester Strain Characterization

Each tester strain characterization plate will be labelled with the following information: tester strain batch number, the letters "AMP" (abbreviation for ampicillin), a plus sign, the letters "CV" (abbreviation for crystal violet), and the date of the experiment. An example of this method would be:



Tester Strain Titers

Each tester strain titer plate will be labelled with the following information: tester strain batch number, total dilution factor, and date of the experiment. An example of this method would be:

$$\underbrace{\text{TA100 5/25}}_{\substack{\text{batch}\\ \text{number}}} \quad \underbrace{2 \times 10^{7}}_{\substack{\text{total}\\ \text{dilution}\\ \text{factor}}} \quad \underbrace{\text{5/26}}_{\substack{\text{date}\\ \text{of}\\ \text{experiment}}}$$

Mutagenesis Experiment

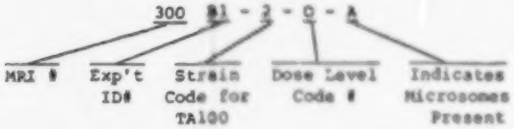
Each plate in the mutagenesis experiment will be labelled with the test article MRI code number, the letter B which will identify the plate as being used in a mutagenesis plating experiment, a number which will indicate the number of times the test article has been tested in the mutagenesis assay, a tester strain code number, a test article dose level number, and the letter "A" if the plate contains microsomes. In those experiments where microsomes from more than one species are used, the "A" will be preceded by the first letter of the species name, e.g. R for rat, H for hamster. The code numbers for the tester strains are:

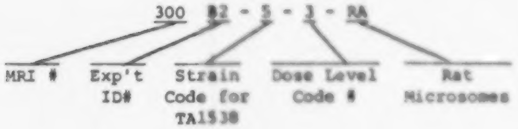
1 - TA98
2 - TA100
3 - TA1535
4 - TA1537
5 - TA1538

The dose level code numbers are:

0 - solvent
5 - fifth test article dilution
4 - fourth test article dilution
3 - third test article dilution
2 - second test article dilution
1 - first test article dilution

An example of this labelling method would be:



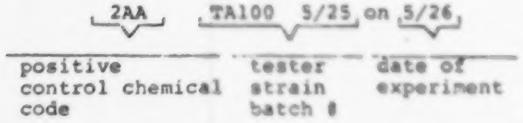


Positive Controls

Each positive control plate will be labelled with the positive control chemical code, the tester strain batch number, the word "on", and the date of the experiment. The codes for the standard positive control chemicals will be:

- 2AA; 2-Aminoanthracene
- PS; 1,3-propane sultone
- 9-AAD; 9-aminoacridine
- 2NF; 2-nitrofluorene

An example of this labelling method is:



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Colony Counting

All colonies will be counted with a BioTrans II automated colony counter whenever possible. If automated counting is not possible then colony counts will be made by hand. Revertant colony counts will be recorded on Forms WL-160, 161 and 162. Data from replicate platings will be averaged and the standard deviation will be calculated and recorded.

Test Article Stability in the Test System

The stability of the test article under the actual experimental conditions used in this study will not be determined by EG&G Mason.

All test article stock solutions will be freshly prepared immediately before their use in each procedure.

Toxicity Determination of Test Articles

Each test article will be checked for toxicity to the tester strains up to a concentration of 10 mg/plate for solids and up to 10 µl/plate in the case of liquids. Eight serial half-log dilutions of the test article will be plated with TA100 on minimal agar plus 10XSA and on minimal agar plus 1XSA. Equal numbers of cells will be seeded on each plate in the presence of the test article. The survival of an appropriately diluted TA100 culture on the 10XSA supplemented plates will be determined by comparing numbers of colonies on the solvent control with those on the plates containing test article. Toxicity on the 1XSA supplemented plates is detectable by a decrease in the number of revertant colonies occurring per plate and by a thinning or disappearance of the background bacterial lawn. The highest concentration of test article used in the broad range mutagenesis assay will be that which gives a detectable reduction of spontaneous revertants on the 1XSA supplemented plates and/or a reduced survival on the 10XSA

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supplemented plates. If no toxicity is observed, then the highest dose level used in the mutagenesis assay will be 10 mg/plate unless:

1) The test article exhibits limited solubility or is not uniformly dispersible in the solvent of choice.
2) The test article precipitates heavily in the top agar.
3) There is insufficient test article available to either demonstrate toxicity or achieve a maximum dose level of 10 mg/plate.
4) The study director indicates an alternate top dose level.

The results of the preliminary toxicity study will be recorded on Form No. WL-162.

Plating Procedures for Mutagenesis Plate Incorporation Assay

The test article will be prepared immediately before its use in the mutagenesis assay. Five doses of the test article will first be plated will all five tester strains (TA98, TA100, TA1535, TA1537, TA1538) with metabolic activation, after which they will immediately be plated on all tester strains without metabolic activation. All positive controls, solvent controls, and test article dilutions will be plated in triplicate. Without metabolic activation, 50 µl of tester strain and 50 µl of solvent or test article will be added to 2.5 ml of molten top agar at 45°C. With metabolic activation, 50 µl of tester strain, 50 µl of solvent or test article, and 0.5 ml of S-9 mix will be added to 2.0 ml of molten top agar at 45°C. To achieve the desired maximum concentration per plate of the test article it will sometimes be necessary to plate 200 µl or 100 µl aliquots instead of the usual 50 µl aliquots. Appropriate solvent controls will be included in these instances. After vortexing, the mixture will be poured onto the surface of 25 ml of bottom agar contained in a 15x100 mm plastic petri dish. After the top agar has solidified, the plates will be inverted and incubated for 48hr at 37°C.

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Positive Controls

Positive controls will be run with each tester strain. All combinations of positive controls and tester strains are listed on Form No. WL-161.

Tester Strain Titers

Tester strain titers will be determined by viable count assays of nutrient agar plates. The number of cells plated per plate will be reported on Form No. WL-160.

Preparation and Storage of Liver Microsomal Enzymes

Liver microsomal enzymes will be routinely prepared from male Sprague-Dawley rats that have been injected with Aroclor 1254 at 500 mg/kg. The Aroclor will be diluted in corn oil to a concentration of 200 mg/ml. Five days after their i.p. injection with the Aroclor, the rats will be sacrificed by decapitation, and their livers will be excised. The rats will be denied access to food for 12 hours immediately preceding sacrifice.

The preparation of the microsomal enzyme fraction will be carried out with sterile glassware and solutions at 0-4°C. The liver from each rat will be excised and placed in 20 ml of 0.15M KCl contained in a pre-weighed beaker. After weighing the liver, it will be transferred to another beaker containing 3 volumes of 0.15M KCl (3ml/g of wet liver) where it will be minced with sterile scissors. The minced liver will be homogenized in a Potter-Elvehjen apparatus with a teflon pestle. The homogenate will be centrifuged at 9000 x g for 10 minutes in the SS-34 rotor of a Sorvall SS-3 centrifuge. The supernatant (referred to by Ames as the S-9 fraction) will be decanted, and small aliquots will be distributed into freezing ampules which will be stored in liquid nitrogen.

One ml of the microsomal enzyme reaction mixture (S-9 mix) which is added to the soft agar overlay will contain the following components:

S-9	0.10 ml
0.4M $MgCl_2$	0.02 ml
1.65M KCl	0.02 ml
0.04M NADP	0.10 ml
0.05M Glucose-6-phosphate	0.10 ml
1.00M NaH_2PO_4, pH 7.4	0.10 ml
H_2O	0.56 ml
	1.00 ml

Evaluation of Mutagenesis Assay Data

For a test article to be considered positive, it must cause at least a doubling in the observed revertants per plate of at least one tester strain. This increase in revertants per plate must be accompanied by a dose response to increasing concentrations of the test article.

Contingencies

In cases where the amount of test article available for testing is limited, a preliminary toxicity study will be conducted as described above.

The final experimental design of the mutagenesis assay will depend upon the toxicity level of the test article. If there is sufficient toxicity present, the standard mutagenesis assay will be conducted as described in the above protocol. If not, then the final experimental design of the mutagenesis experiment will be determined after discussions between the Study Director and the Sponsor's Study Coordinator.

Archives

All experimental records of the study will be maintained in the EG&G Mason Research archives located at 1530 East Jefferson Street, Rockville, Maryland 20852.

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Preliminary Toxicity Determination of Test Article

Experiment Number ______

Study Number ______

Test Article Identification ______

Test Compound Concentration µ /Plate	TA100 Viable Count/ Plate	TA100 Revertants/ Plate	TA100 Background Bacterial Lawn
			*

* See Key on the Following Page

Date Plated ______

Colonies were ______ Counted

Eight serial half-log dilutions of the test compound are plated with TA100 on minimal agar plus 10XSA and on minimal agar plus 1XSA. Equal numbers of cells are seeded on each plate in the presence of the test compound. The percent survival of an appropriately diluted TA100 culture on the 10XSA supplemented plates is determined by comparing numbers of colonies on the solvent control with those on the plates containing test compound. Toxicity on the 1XSA supplemented plates is detectable by a decrease in the number of revertant colonies occurring per plate and by a thinning or disappearance of the background bacterial lawn.

Form No. WL-162 1.0 7/80
5/5/80

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SALMONELLA MUTAGENESIS ASSAY

______ Study Number	______ Test Article Identification	______ Dose Range
______ Experiment Number	Solvent	______ Concentration (µ per plate) ______

Strain: ______ Date Plated: ______ No. of Cells Seeded: ______ x10^8 Metabolic Activation: Rat Liver Colonies were ______ counted	Revertants per Plate								
	Averaged Revertants								
	Standard Deviation								
Strain: ______ Date Plated: ______ No. of Cells Seeded: ______ x10^8 Metabolic Activation: None Colonies were ______ counted	Revertants per Plate								
	Averaged Revertants								
	Standard Deviation								

Form No. WL-160
5/5/80

1.0
7/80

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SALMONELLA MUTAGENESIS ASSAY

Positive Controls

______ Study Number ______ Experiment Number ______ Test Article Identification

Date Plated	Strain	Chemical	Concentration per plate	Metabolic Activation	Revertants/Plate			Averaged Revertants per plate	S.D.
	TA98	2-Aminoanthracene	1.0 µg	Rat Liver					
	TA98	2-Nitrofluorene	10.0 µg	None					
	TA100	2-Aminoanthracene	1.0 µg	Rat Liver					
	TA100	1,3-Propane Sultone	0.04 µl	None					
	TA1535	1,3-Propane Sultone	0.04 µl	None					
	TA1537	9-Aminoacridine	75 µg	None					
	TA1538	2-Nitrofluorene	10.0 µg	None					

Form No. WL-161
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Colonies were ______ counted

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APPENDIX H

Laboratory: Genetic Bioassay Branch
Genetic Toxicology Division
Health Effects Research Laboratory
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711

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Submitter: Dr. Larry D. Claxton
Research Biologist
(919)541-3848

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Date of submission: November 1, 1980

Omissions: Part II, section 6-8
Part III
Appendices
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THE GENETIC BIOASSAY BRANCH
PROTOCOL FOR BACTERIAL MUTAGENESIS
INCLUDING SAFETY AND QUALITY ASSURANCE PROCEDURES

by

Larry D. Claxton
Michael Kohan
Ann C. Austin
Carol Evans

Genetic Bioassay Branch
Genetic Toxicology Division
Health Effects Research Laboratory
U.S. Environmental Protection Agency
Research Triangle Park, North Carolina 27711

HEALTH EFFECTS RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
RESEARCH TRIANGLE PARK, NORTH CAROLINA 27711

DISCLAIMER

This report has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

DISCLAIMER

This report has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

This user's manual provides an overview of bacterial mutagenicity protocols and the detailed protocol for the Salmonella plate incorporation assay (Ames test) used by the Health Effects Research Laboratory. In addition, it discusses quality assurance, data computerization, and data interpretation methods used by the Laboratory.

F. G. Hueter, Ph.D.
Director
Health Effects Research Laboratory

ABSTRACT

Researchers often choose microbial tests for use in genetic toxicology studies because they are rapid and inexpensive, and because they supply dose-responsive data. This user's manual documents the test methods used extensively by the Health Effects Research Laboratory (HERL), Research Triangle Park, North Carolina over the past 4 years.

The first part of the report describes the basic protocol (the plate incorporation test), special precautions, and specific modifications of the assay routinely used in HERL studies. The second part presents HERL's stepwise protocol for the plate incorporation test and protocols for several other biological assays, including the spot test, the forward mutation assay procedure, and the fluctuation test. The last portion of the report provides the necessary information for experiment planning and provides sample information forms and computerization procedures necessary for reporting experimental results.

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PART I

OVERVIEW OF PROTOCOLS AND SAFETY PROCEDURES

SECTION I

INTRODUCTION

A variety of short-term genetic bioassays are currently available for mutagenicity testing of chemical compounds. From these assays, the microbial plate incorporation tests are often chosen for use in genetic toxicology studies because they are rapid and inexpensive, and because they supply dose-responsive data. However, some of the basic protocols for these tests cannot determine mutation frequencies.

Part I of this report describes the basic protocol (the plate incorporation test), special precautions, and specific modifications of the assay routinely used in Health Effects Research Laboratory (HERL) studies. This portion of the report also describes the applications of this assay to complex mixtures such as industrial and mobile source pollutants, and presents an overview of methods for chemically extracting and concentrating pollutant samples.

Part II presents HERL's stepwise protocol for the plate incorporation test as well as protocols for several other biological assays, including the spot test, the forward mutation assay procedure, and the fluctuation test.

The last portion of the report, Part III, provides information necessary for experiment planning and includes sample information forms and computerization procedures necessary for reporting of experimental results.

SECTION 2

BASIC *SALMONELLA TYPHIMURIUM* PLATE INCORPORATION TEST

The reference protocol for the *Salmonella typhimurium* plate incorporation test is described by Ames *et al*. (1). Part II gives the stepwise procedure used by the author of this report. The outline of the basic procedural protocol is very simple (Figure 1-1). A specific amount of the test chemical and a culture of the indicator bacterial strain are added into a melted soft agar overlay. The mixture is then poured onto a minimal media plate that will select for bacteria having undergone mutation. The plate is incubated 48 to 72 h at 37°C and the number of colonies per plate then counted. This procedure is repeated for each dose of the chemical, for each bacterial strain, for each routine modification (e.g., with and without microsomal activation), for each needed control, and for each replication. The procedure generates approximately 210 data points (plate counts) for each chemical analyzed.

The *Salmonella* procedure detects reverse mutations that occur in histidine-requiring strains of *Salmonella typhimurium* developed by Dr. Bruce Ames (1). When these strains undergo a reverse mutation to prototrophy, the revertants can be detected on a minimal nutrient medium that lacks histidine. Although the reverse mutation system detects only reverse mutations, forward mutation detection systems are not limited to a specific type of genetic event

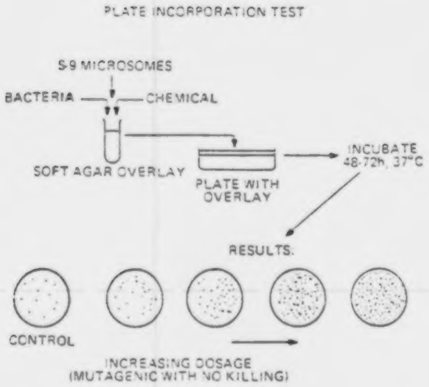

Figure 1-1. Outline of the basic Salmonella typhimurium plate incorporation test with mammalian activation.

(e.g., a frameshift or a point mutation). Since screening systems need to identify a variety of genetic mutational events, several different Salmonella strains have been developed. Each strain detects a specific type of mutation and incorporates other changes needed to increase its sensitivity. Five strains are commonly used (Table 1-1), three of which (TA1537, TA1538, and TA98) detect frameshift mutations. TA1537 detects compounds such as 9-aminoacridine. TA1538 and TA98 both detect compounds such as 2-nitrofluorene, but differ in that TA98 contains an R-factor plasmid and is more sensitive to some frameshift mutagens than TA1538. Chemicals that produce base-pair substitutions, especially alkylating agents, are detected in TA1535. Strain TA100 was developed from TA1535 by the addition of the R-factor plasmid.

The R-plasmid causes a higher spontaneous mutation rate and can detect chemicals that normally yield frameshift mutations.

TABLE 1-1. CHARACTERISTICS OF FIVE *SALMONELLA TYPHIMURIUM* TESTER STRAINS

Strain	*his*-Mutation[*]	*rfa*[†]	*uvr* B[‡]	R-factor plasmid	Spontaneous revertants/plate
TA1535	*his* G46	Yes	Yes	No	5-50
TA1537	*his* C3076	Yes	Yes	No	2-25
TA1538	*his* D3052	Yes	Yes	No	5-40
TA98	*his* D3052	Yes	Yes	Yes	15-35
TA100	*his* G46	Yes	Yes	Yes	80-200

[*] *his*-Mutation: Histidine mutation incorporated
[†] *rfa*: Mutation for deficiency in bacterial cell wall
[‡] *uvr* B: Ultraviolet repair gene B deletion

In addition to the histidine locus mutation, these *Salmonella* strains carry an *rfa* mutation that creates a deficiency in the bacterial cell wall lipopolysaccharides. This deficiency increases the cell's permeability to large molecules. The *uvr* B mutation was incorporated into these strains to increase their susceptibility to several classes of mutagens by decreasing genetic repair. The R-factor plasmid found in strains TA98 and TA100 increases further the sensitivity of the tester strains by participating in a type of error-prone repair. This plasmid is detected by ampicillin resistance. The five strains also all differ in the number of spontaneous revertants per plate generally found. Table 1-1 summarizes the general characteristics of the strains.

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SECTION 3

MAMMALIAN METABOLIC ACTIVATION

Since some compounds that are carcinogenic for mammals are not mutagenic for bacteria, carcinogenic potential in humans cannot always be predicted by bacterial systems. Therefore, a mammalian metabolic activation system was added to the basic Salmonella assay to allow for some of the metabolites as well as the agent itself to be tested for mutagenicity. The activation system described by Ames (1) is usually used and consists of a 9000 x g supernatant of a rat liver homogenate. Generally, Aroclor is administered to the rats from which the homogenate is prepared to induce the hepatic enzymes referred to collectively as mixed function oxygenases (MFO). An induced MFO system potentiates increased metabolite levels and a corresponding increase in revertant colonies due to mutagenic metabolites.

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SECTION 4

PRECAUTIONS AND PROCEDURAL MODIFICATIONS

Although the reference protocol (1) has been described in great detail, protocols with modifications and differing quality control procedures have been presented in the literature as "standard protocols" (2, 3). The modifications and quality assurance procedures used by the author of this report are presented, as closely as possible, in the sequence encountered in the testing protocol.

CHECKING OF TESTER STRAINS

Upon their receipt and at regular intervals, the tester strains are checked for ultraviolet (UV) sensitivity, ampicillin resistance, and crystal violet killing. If these parameters indicate a mixed culture or give an unacceptable response, new single colony isolates are grown from a stock culture tube. The strains are also checked for spontaneous and induced mutant levels at the time of creating master plates.

CRITERIA FOR PROPER CULTURE

Bacterial strains, culture media, and testing conditions may vary between culture preparations; the bacterial cultures used for testing vary accordingly. Three conditions, however, are always met for the routine screening of chemicals. First, a single colony isolate is always used to initiate the culture. This helps to insure genetic uniformity of the broth culture. Secondly, the test culture always has a concentration of 1-2 x 10^9 viable cells per mL. This concentration is assured by using the culture in the early portion of the stationary growth phase. Next, if the cells are not used immediately upon harvesting, they are stored in an ice water bath.

MAMMALIAN ACTIVATION SYSTEM

This protocol uses the 9000 x g supernatant (S-9) obtained from rat liver induced with Aroclor 1254. This S-9 mixture is prepared by the supplier from Charles River CD-1 rats weighing between 180 and 200 g, using the preparation procedures of Ames (1). The protein concentration is determined by the method of Lowery (4) and recorded. Since the level of enzymatic activity can vary with each batch of S-9, various concentrations of the S-9 are tested within the bioassay against standard concentrations of two or three known mutagens. For each mutagen, the S-9 concentration that provides the maximum number of revertants per plate is determined; the concentrations of the two maximum responses are averaged to provide the S-9 concentration used for testing. The three mutagens used are benzo(a)pyrene, 2-anthramine, and 7,12-dimethylbenzanthracene.

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SUBSTANCE TESTED

In order to assure proper testing, several guidelines are followed. Each sample is tested at a minimum of five doses at half-log intervals to survey a broad range of doses. Compounds that demonstrate little or no toxicity are tested in the 5 to 10 mg per plate range before being reported as negative. The solubility of the test substance is noted both when placed into the solvent and into the agar overlay. Agents which precipitate or demonstrate an apparent loss of highly volatile substances are retested with modified conditions. Although more refined procedures are being developed, volatile substances presently are tested in sealed bags or desiccators. Indication of the toxicity is recorded, and if clearing of the test plates is seen at all doses, lower doses are tested to assure that the effect observed is not due to toxicity. When toxicity does result, some of the bacteria may lyse and contribute histidine to the media. Therefore, cross-feeding may result and colonies that are not revertants may be found. These colonies are usually small pinpoint colonies, though not always. Other substances, especially complex mixtures and technical grade chemicals, may contain either histidine or histidine-like acting components. Colonies from representative plates are transferred to minimal media plates without histidine to determine whether or not they are true revertant colonies.

WITHIN EXPERIMENT CONTROLS

All tests contain appropriate positive, negative, and sterility controls. Table 1-2 records positive controls for the five commonly used tester strains.

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The negative control consists of the entire system without the compound to check spontaneous mutation rate. The activation mix and test substance are both checked for sterility.

TABLE 1-2. CONTROL MUTAGENS FOR THE FIVE COMMONLY USED TESTER STRAINS OF SALMONELLA TYPHIMURIUM

Strain	Without activation (-S-9)	With activation (+S-9)
TA1535	MNNG, or sodium azide, or methylmethane sulfonate	2-Anthramine
TA1537	9-Aminoacridine	2-Anthramine
TA1538	2-Nitrofluorene, or 4-nitro-o-phenylenediamine	2-Anthramine
TA98	2-Nitrofluorene, or hycanthone methanesulfonate	2-Anthramine
TA100	MNNG, or sodium azide, or MMS, or nitrofurantoin	2-Anthramine

SPECIAL PROCEDURES FOR COMPLEX MIXTURES

Complex mixtures present a variety of technical difficulties in the Salmonella test. Most complex mixtures are direct environmental pollutants which are sampled in various ways. Some of these samples are very low in organics, while others are entirely organic.

The mutagenic activity of a complex mixture may not be detectable because of problems such as too high a dilution, presence of a sample matrix which prevents release of the mutagens into the bacterial media (as in the case with

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certain carbonaceous particulate samples), or the presence of highly toxic compounds in the mixture which result in sufficient bacterial killing to prevent detection of bacterial mutants. To overcome these problems, most of the complex mixtures tested are concentrated, extracted, or fractionated prior to bioassay. Examples of applications of these methods are described below.

Concentration

Various methods are used to concentrate the pollutant samples. If the sample is in particulate or precipitate form, filtration is used to concentrate it. The type of filtration used depends upon the type of sample. For example, air particulates are generally filtered by the use of pallflex filters and glass fiber filters, and are concentrated by impaction devices and electrostatic precipitation.

Extraction

With particulates, the active organic compounds condense onto a variety of materials including silica and carbon. Since bacteria cannot ingest most of these solid materials (as some mammalian cells are able to), these organics are extracted from the solid matter. Samples for these studies are extracted by a variety of processes (e.g., in a Soxhlet apparatus at 45°C with dichloromethane). Particle mass is determined both before and after extraction, and the percent of organic mass per particle mass is calculated. The dichloromethane-organic extract mixture is blown to dryness or near dryness under dry nitrogen. After weighing, the organics are dissolved in dimethyl sulfoxide (DMSO) or some other appropriate solvent. All samples are tested in a solvent compatible with the biological test system.

SAFETY PRECAUTIONS

The following is a listing of some of the safety procedures established within the author's laboratory:

- All samples for testing and positive control chemicals are housed in a special weighing and storage area. These samples and chemicals are recorded and logged in and out.
- All weighings and measurements of stock chemicals are handled in a special weighing room within glove boxes which are vented to the outside through charcoal filters located on the roof (see Part II, Section 1).
- Whenever possible, incubators, storage cabinets, and refrigerators are vented, and upon opening any of these units, the air within the unit is exhausted.
- All work is performed in laminar flow exhaust hoods or total exhaust biological hoods (see Part II, Section 1).
- All work is completed in designated areas, and personnel are properly clothed. (Proper clothing includes a disposable gown or jump suits, two pair of latex gloves, safety glasses, safety shoes, and shoe covers. Each employee is instructed in the proper use of the clothing.)
- No mouth pipetting is allowed for any procedure.
- No eating, drinking, smoking, chewing of gum, or application of cosmetics is allowed within the laboratory. No food or drink may enter the laboratory.
- If an EPA safety official or the supervisor determines the noise level high enough to warrant ear protection, an ear protection device is worn by all personnel at any time at which the noise reaches this level (e.g., when hoods are operating).
- Each phone is posted with the emergency number (#2900) and the room number in which the phone is located. A copy of the emergency reporting procedure is also adjacent to each phone.

- Each employee must learn and follow all emergency evacuation procedures.
- All waste is double-bagged and placed in drums or special containers which are sealed and sent by a contractor to special areas for disposal or incineration.

DATA INTERPRETATION

Information on controls (both positive and negative), sterility when contamination is reported, and the replication of plates at each dose and replication of each test is either reported for each sample or can be assumed to be within normal limits. This is also true for the number and range of doses used and the toxicity of the sample.

The determination of definite positives in the author's laboratory is based on the following criteria (Statistical tests are based upon the model by Stead et al. [5]):

- The data must not vary significantly from a Poisson distribution ($p > 0.01$) (5).
- The data must be acceptable by the test of adequacy ($p > 0.01$) (5).
- The test for mutagenicity (i.e., the slope of the curve) must be significant ($p < 0.01$) (5).
- At least a twofold increase must have occurred over spontaneous levels at one or more doses.
- A dose-response curve with some type of "regular curve" must have been seen (e.g., a single high response would not be acceptable).
- All controls must have given expected responses.
- Histidine cross-feeding and/or contamination must not have been shown to occur.

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Data fitting all of the above criteria are clearly positive. However, a positive response may occur in which all of the above criteria do not apply. The following are the criteria used as guidelines by the author in these cases:

- In all cases the controls give expected responses and the histidine cross-feeding and/or contamination is never shown to occur.
- When the data depart significantly from a Poisson distribution ($p < 0.01$), but all other items hold, and a single dose or plate causes deviation from the Poisson distribution, the response can be considered positive.
- When the test for adequacy shows a significant departure from the expected response ($p < 0.01$), but all other items hold, and a single dose or plate causes this significance, the response can be considered positive.
- If no dose gives a twofold increase over spontaneous but all other items hold, the response can be considered a borderline positive.

In each of these cases quantitative comparisons are questionable and, if possible, the tests should be repeated.

Since most positive results are easily distinguished, experiments are designed to give some definition to a negative result. The following are the minimal testing requirements used to define a negative test response:

- A minimum of 5 doses at half-log intervals with the highest dose being highly toxic or presented at a minimum of 5 mg per plate
- Spontaneous and positive controls done at least in duplicate and providing the expected response
- Positive controls (in duplicate) for any bacterial/microsomal activation combination used are within normal ranges.
- At least two replicates per dose

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- At least one replicate experiment, if possible, done with a narrower dose range
- Tested in TA1535, TA1537, TA98, and TA100
- A negative response with the sterility controls
- Restreaked colonies grew on histidine-free media.
- Lack of any indication of mutagenicity (e.g., With the Stead model (5), the test for mutagenicity is not significant [$p > 0.01$]).

A variety of ways to present summarized data is available. deSerres and Shelby, however, suggest that summarized data not be presented without supporting data (6). The following is a listing of some of the ways to summarize and compare data:

- Fold increase or mutagenic index: Average plate counts divided by average spontaneous count for the same bacterial strain
- Net increase in revertants: Average counts or individual counts minus average spontaneous count. (Generally considered unacceptable)
- Specific activity: Slope of the linear portion of the dose-response curve
- Specific response activity: Calculated dose needed to give a specific number of revertants per plate (e.g., 200 rev/plate) for a specific strain
- Maximum response: Maximum number of revertants per plate seen
- Model slope: Revertants/plate/dose as calculated from a curve that models the test response

These methods are used to compare the results of a series of tests, but do not necessarily reveal the adequacy or positiveness of the results. Several methods for examining Salmonella plate test data have been or are being published. The model by Stead et al. supplies the most stringent criteria for acceptance of data (5).

PART II

HEALTH EFFECTS RESEARCH LABORATORY STEPWISE EXPERIMENTAL PROTOCOLS

SECTION 1

INITIAL EQUIPMENT LIST

PERMANENT MAJOR EQUIPMENT

Delineated below is a list of equipment essential for successful completion of biological bioassays and which must be maintained at all times to assure accurate test results:

- Biological safety cabinet (hood)
- Autoclave
- Incubators
- Refrigerator(s) and freezers
- Balances (both an analytical and a top-loader balance)
- Bacterial colony counter (any standard model)
- Water baths (for media preparation and maintaining overlay tubes)
- Shaker heater or water bath (for culturing media and for preincubation test)

The biological safety cabinet (hood) must be designed to protect both personnel and the work area. The hood's intake air must be high-efficiency, HEPA-filtered, and have no greater than 30% recirculation. Airflow over the

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work surface is to be directed away from personnel and exhausted. All exhaust air should flow through a trap appropriate for removing organic materials.

The autoclave chosen must be of good quality and be able to maintain standard autoclave conditions. Since the autoclave is used to house large reagent bottles and flasks, size is also a necessary consideration.

Forced air incubators are preferable to other available types because they allow better temperature control. All incubators used must maintain a uniform temperature (37°C ± 5°C) throughout the chamber. Air from the incubators should be directly or indirectly exhausted.

Refrigerators and freezers must be lockable. Any refrigerators used for strong solvents or organic materials must be explosion-proof. Freezer temperature for bacteria storage is -80°C.

OPTIONAL MAJOR EQUIPMENT

To increase the accuracy of test results, other major equipment may be needed, including:

- Automatic colony counter
- Automatic plate pourer
- Dri-block heaters for 13 x 100 mm test tubes

The range of reliability and any needed correction curves must be established for the automatic colony counter. The counter must also be checked

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routinely for accuracy. The automatic plate pourer must be able to fill plates with a precise amount of media under sterile conditions. Dri-block heaters for 13 x 100-mm test tubes may be used in place of a water bath for overlay tubes.

MINOR EQUIPMENT

The list below contains minor equipment necessary for completion of *Salmonella* tests:

- Automatic pipettes
- Glassware
- Plastic bag sealer
- Automatic repipette

Separate pipettes are needed for the various volumes used in the *Salmonella* test. Glassware used should include Erlenmeyer flasks (4-L, 2-L, 500-mL and 50-mL), reagent bottles (1-L and 200-mL), and grauuated cylinders (1-L, 100-mL, and 50-mL). An automatic repipette is useful for S-9 and bacterial broth. The repipette must be calibrated and routinely checked.

DISPOSABLE EQUIPMENT

All of the following equipment is disposable:

- Automatic pipette tips
- Plastic bags for incubation of plates
- Hazardous waste bags
- Latex gloves
- Petri dishes (15 x 100-mm, sterile)
- Test tubes (13 x 100-mm)
- Test tube caps for 13 x 100-mm tubes

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- Pipettes (TD, disposable, glass; 1-mL, 2-mL, 5-mL, and 10-mL)
- Personnel gowns
- Table top covers

SECTION 2

MEDIA PREPARATION

50X VBME STOCK SALTS SOLUTION

To prepare 50X VBME stock salts solution, measure out 600 mL deionized water into a clean 2-L Erlenmeyer flask. Add magnet stirring bar and heat on a hot plate, with stirring. After water is moderately hot (about 45°C), add the following chemicals slowly in the following order:

(1)	Magnesium sulfate ($MgSO_4 \cdot 7H_2O$, MW = 246.5)	10 g
(2)	Citric acid monohydrate ($HOCCOOH\,[CH_2COOH_2]_2 \cdot H_2O$, MW = 210.14)	100 g
(3)	Potassium phosphate dibasic anhydrous (K_2HPO_4, MW = 174.18)	500 g
(4)	Sodium ammonium phosphate ($Na[NH_4]HPO_4 \cdot 4H_2O$, MW = 209.07)	175 g

After all chemicals are dissolved, pour the solution into a glass 1-L graduated cylinder and bring to 1000 mL volume by adding deionized water. Then filter the solution through a Buchner funnel (with Whatman #1 filter paper) into a vacuum flask. After filtering is complete, pour 500 mL of the solution into each of two clean 1-L Wheaton bottles labeled with 50X VBME solution and date prepared. Autoclave the two bottles, loosely capped, for 25 min on slow exhaust. After the bottles and their contents are well cooled, tighten the caps and place the bottles in cabinet. Do not store for more than two months.

HISTIDINE/BIOTIN SOLUTION

To prepare the histidine/biotin solution, first measure out 1000 mL deionized water and pour into a clean 2-L Erlenmeyer flask. Cover the flask with tinfoil, then autoclave for 30 min on slow exhaust. After the water in the flask cools slightly, add 0.0525 g l-histidine and 0.0976 g biotin and shake vigorously to dissolve.

Turn hood on and allow to run for a few minutes. Pour the His/Bio solution into a sterile 1000-mL Wheaton bottle labeled with "His/Bio solution, sterile." Also label the bottle with the date prepared. Store in refrigerator for no longer than one week.

BIOTIN SOLUTION

The biotin solution should be prepared as the above solution except the biotin solution uses only 0.0976 g biotin and no histidine. Label as Bio solution.

PREPARATION OF VBME PLATES (APPROXIMATELY 100 PLATES)

Materials needed to prepare 3 L of media are:

- One 4-L Erlenmeyer flask
- One 500-mL Erlenmeyer flask
- Difco agar
- Dextrose
- His/Bio solution
- 50X VBME solution
- One sterile glass 100-mL graduated cylinder
- One sterile glass 50-mL graduated cylinder

Into a 4-L flask add 2655 mL of deionized water and 45 g of Difco agar. Cover the flask with tinfoil and shake gently. Then place the flask on a hot plate and heat, with stirring, until agar dissolves. Watch carefully to avoid agar burning to the bottom of the flask.

Pour 200 mL of deionized water into a 500-mL flask. After adding 60 g of dextrose, cover with tinfoil and shake gently. Place in a 55°C water bath to dissolve dextrose. After agar and dextrose are dissolved, autoclave both flasks for 35 min using slow exhaust. After flasks cool slightly, add dextrose solution to agar.

Place the 4-L flask in a 55°C water bath. After 15 min, measure out 60 mL of 50X VBME solution using a sterile 100-mL graduated cylinder and pour into the flask. Mix and return to water bath. After an additional 15-min wait, add 30 mL of the His/Bio solution using a sterile graduated cylinder into the flask. Mix and return to the 55°C water bath. Using an automatic plate pourer, dispense 30 mL of the media into each 100 x 15-mm petri plate. After agar solidifies, shake off condensation remaining on the lids of the plates.

Incubate the plates (inverted) at 37°C overnight. After examining them for contamination, bag the plates (20 plates per bag). Write the batch number and date on each bag and record the total number of plates made.

PREPARATION OF OTHER NECESSARY SOLUTIONS

Nutrient Broth

Dissolve the ingredients listed below and dispense into desired vessels. Then autoclave.

• Difco bacto nutrient broth	8.0 g
• Sodium chloride	5.0 g
• Distilled water	1.0 L

Nutrient Agar

Mix the ingredients below and autoclave for 15 min. Swirl melted agar to mix and then pour onto plates.

• Difco bacto nutrient broth	8.0 g
• Sodium chloride	5.0 g
• Agar	15.0 g
• Distilled water	1.0 L

Top Agar

Dissolve 6 g bacto-agar and 5 g NaCl completely in 1000 mL deionized water and dispense the mixture into clean screw-capped bottles (100 mL/bottle) or culture tubes (2.1 mL/tube). Autoclave for 15 min at 121°C. Use slow exhaust. Before use, melt in autoclave or microwave and cool to 45°C.

$MgCl_2$ - KCl Solution

Autoclave a solution of 81.32 g $MgCL_2 \cdot 6H_2O$, 123.02 g KCl, and 1000 mL deionized water for 20 min.

Sodium Phosphate Buffer (0.2 M)

Mix and autoclave 7.76 g KH_2PO_4, 20.30 g Na_2HPO_4, and 1000 mL deionized water for 20 min. The pH should be adjusted to 7.4 with sodium hydroxide.

Glucose-6-Phosphate (G-6-P)

Immediately before the experiment, 1.41 mg G-6-P per mL of S-9 mix is added to sterile deionized water to obtain the needed amounts of G-6-P.

Nicotinamide Adenine Dinucleotide Phosphate (NADP)

Immediately before the experiment, 3.06 mg NADP per mL of S-9 mix is added to sterile deionized water to create the needed amounts of NADP. Filter and sterilize the solution before use.

SECTION 3

PREPARATION OF RAT LIVER S-9 ACTIVATION MIXTURE

COMPONENTS OF S-9 MIXTURE

The five components of the rat liver S-9 activation mixture are:

- Salt solution ($MgCl \cdot 6H_2O$ - 0.4 M; KCl - 1.65 M)
- G-6-P (1.41 mg/mL S-9 mix)
- NADP (3.06 mg/mL S-9 mix)
- S-9 (usually between 0.05 and 0.1 mL S-9/mL S-9 mix)
- Ames buffer (KH_2PO_4 - 7.76 g/L; Na_2HPO_4 - 20.3 g/L), pH = 7.4

To dissolve G-6-P and NADP, add sterile deionized water. The volume of water is determined by summing the amount of salt solution, buffer, and S-9 added and subtracting this volume from the total amount of S-9 mix being prepared. Sterile filter the NADP solution through a 0.45-μm filter.

The amount of S-9 used in the S-9 mixture depends upon the activity of each new lot of S-9. But, as stated above, the amount is usually between 0.05 and 0.1 mL S-9/mL S-9 mix.

Buffer always makes up one-half of the S-9 mix.

PROTOCOL FOR PREPARATION OF RAT LIVER S-9 FRACTION

Five days prior to sacrifice, inoculate rats weighing approximately 200 g intraperitoneally with Aroclor 1254 in corn oil (200 mg/mL). The delivered dose is 500 mg Aroclor 1254/kg of body weight. One day prior to sacrifice, take the animals off food, but provide water ad libitum. Sacrifice animals by cervical dislocation 5 days after injection. The following procedures are performed in a vertical laminar flow hood, employing aseptic techniques:

- Remove the livers, mince several times, then transfer to a preweighed beaker on ice containing sterile, cold 1.15% KCl.
- Following removal of all the livers (less than 45 min), reweigh the beakers and record the weight of the livers.
- Rinse the liver fragments several times with sterile 1.15% KCl and further mince until tissue fragments of approximately 2-3 mm^2 are obtained. Rinse the liver mince again several times with sterile 1.15% KCl.
- Following the last KCl rinse, transfer the minced liver to a beaker containing sterile 0.25 M sucrose (on ice). Then rinse the minced liver twice, decanting as much of the last sucrose rinse as possible without losing the liver.
- Add sterile 0.25 M sucrose at a 2:1 ratio (sucrose volume:liver weight).
- Homogenize the minced tissue using a 50-mL Potter-Elvehjen homogenizer and pool. One complete downward and upward stroke is used for homogenization.
- Centrifuge the homogenate at 9000 x g for 25 min at 4°C.
- Decant the supernatant through sterile gauze held on ice. Then aliquot a 2 mL volume of S-9 into individual 3 mL polypropylene freezing vials, and immediately quick freeze using liquid nitrogen. Store at -70°C in a Revco low-temperature freezer.

- Randomly select several vials for quality assurance and protein determinations.
- Record observations on computerized information sheets.

SECTION 4

SALMONELLA TESTER STRAINS

OVERVIEW

Presently there are five standard mutant strains of *Salmonella typhimurium* used for routine testing. These five strains -- TA98, TA100, TA1535, TA1537, and TA1538 -- are obtained by HERL from Dr. Bruce Ames at the University of California, Berkeley. Other specialized strains are described as used for each additional test. The sensitivity of all five tester strains has been enhanced by the deep rough (*rfa*) and *uvr* B mutations. The *rfa* mutation eliminates the polysaccharide side chain of the lipopolysaccharide layer coating the bacterial surface making the bacteria cell wall more permeable to higher molecular weight chemicals. The deletion of the *uvr* B gene causes a loss of the cell's excision repair capabilities. The *uvr* B detection also includes deletion of the biotin (*bio*) gene; therefore, all five tester strains also require biotin as well as histidine. Two of the strains, TA98 and TA100, also contain R-factor plasmids which provide ampicillin resistance. These two strains were developed by transferring plasmids into strains TA1538 and TA1535, respectively. This plasmid seems to increase the sensitivity of these two strains by causing error-prone recombination repair; however, the exact mechanism by which the R-factor enhances mutagenesis is not known. The TA1535 and TA100 strains can detect mutagens which cause base-pair substitutions. The other strains, TA1537, TA1538, and TA98, are capable of detecting various kinds of frameshift mutagens. The TA100 strain is also sensitive to many frameshift mutagens. Dr. Herbert Rosenkranz has developed a series of strains that are nitroreductase-deficient (7). These and other strains are also available and are listed in Table 2-1.

TABLE 2-1. CHARACTERISTICS OF _SALMONELLA_ _TYPHIMURIUM_ TESTER STRAINS

	Additional Mutations					
Strain	_his_-Mutation	LPS	Repair	R	ND	Spontaneous reversions/plate
			Common Tester Strains			
TA98	D3052	rfa	Δuvr B	Yes	No	15-75
TA100	G46	rfa	Δuvr B	Yes	No	80-200
TA1535	G46	rfa	Δuvr B	No	No	5-50
TA1537	C3076	rfa	Δuvr B	No	No	2-25
TA1538	D3052	rfa	Δuvr B	No	No	5-40
			Additional Tester Strains			
TA98-FRI	D3052	rfa	Δuvr B	Yes	Yes	
TA100-FRI	G46	rfa	Δuvr B	Yes	Yes	
TA98/NR	D3052	rfa	Δuvr B	Yes	Yes	
TA98/1, 8 DNP$_6$	D3052	rfa	Δuvr B	Yes	Yes	
TA98/NR/1, 8 DNP$_1$	D3052	rfa	Δuvr B	Yes	Yes	
TA98/NR/1, 8 DNP$_2$	D3052	rfa	Δuvr B	Yes	Yes	
TA98/NR/1, 8 DNP$_5$	D3052	rfa	Δuvr B	Yes	Yes	
TA98/1, 8 DNP$_6$/NR$_1$	D3052	rfa	Δuvr B	Yes	Yes	
TA98/1, 8 DNP$_6$/NR$_8$	D3052	rfa	Δuvr B	Yes	Yes	
TA98/1, 8 DNP$_6$/NR$_{11}$	D3052	rfa	Δuvr B	Yes	Yes	
TA98/1, 8 DNP$_6$/NR$_{12}$	D3052	rfa	Δuvr B	Yes	Yes	
TA98 NIR 301	D3052	rfa	Δuvr B	Yes	Yes	
TA98 NIR 302	D3052	rfa	Δuvr B	Yes	Yes	
TA98 NIR 303	D3052	rfa	Δuvr B	Yes	Yes	
TA98 NIR 304	D3052	rfa	Δuvr B	Yes	Yes	

(continued)

TABLE 2-1. (continued)

	Additional Mutations					
Strain	*his*-Mutation	LPS	Repair	R	ND	Spontaneous reversions/plate
Additional Tester Strains						
TA100/NR	G46	rfa	Δuvr B	Yes	Yes	
98ND	G46	rfa	Δuvr B	Yes	Yes	
100ND	G46	rfa	Δuvr B	Yes	Yes	
TA1978	D3052	rfa	+	No	No	
TA1977	C3076	rfa	+	No	No	
TA1975	G46	rfa	+	No	No	
TA1964	D3052	Δgal	Δuvr B	No	No	
TA1532	C3076	Δgal	Δuvr B	No	No	
TA1530	G46	Δgal	Δuvr B	No	No	
TA2641	D3052	Δgal	Δuvr B	Yes	No	
TA2631	G46	Δgal	Δuvr B	Yes	No	
TA2637	C3076	rfa	Δuvr B	Yes	No	
TA1534	D3052	+	Δuvr B	No	No	
TA1952	C3076	+	Δuvr B	No	No	
TA1950	G46	+	Δuvr B	No	No	
TA2410	G46	+	Δuvr B	Yes	No	
TM35	+	rfa	Δuvr B	No	No	
TM67	+	rfa	Δuvr B	Yes	No	
Parental Strains						
LT2	+	+	+	No	No	
G46	G46	+	+	No	No	
C3076	C3076	+	+	No	No	
D3052	D3052	+	+	No	No	

ROUTINE CHECK OF STRAINS

Each strain should be checked for five specific characteristics on a routine basis. These checks are essential, especially in the case of the unstable plasmid-containing strains TA100 and TA98. Characterization of the tester strains should be performed each time the strains are grown from frozen permanents, according to the procedure discussed below.

Histidine Requirement

Add a nutrient broth culture (0.1 mL) of each strain to an overlay agar tube and pour onto a minimal media plate both with and without histidine (0.1 mL of 0.1 M l-histidine) and biotin (0.1 mL of 0.5 mM biotin). After 72 h at 37°C, growth should be observed on the plates with histidine and biotin. No growth should appear on the non-supplemented plates; however, spontaneous revertant colonies from the overnight broth culture will appear upon this non-supplemented media.

Spontaneous Reversion

The spontaneous reversion rate should be within the range recorded in Table 2-1. A time course record of the results should be kept. The method is as follows:

- Melt top agar in autoclave or microwave oven. Pipet 2.0 mL into each sterile, capped, 13 x 100-mm culture tube. The culture tubes should be preheated and maintained at 45°C in a water bath or heating block.
- For each tester strain, add 0.1 mL of broth culture to a culture tube, mix, and pour onto the surface of a minimal agar plate. Spread the top agar uniformly over the surface of the minimal agar by gently rotating the plate. Repeat this process three times for each strain.
- An optional procedure is to repeat the second step with the addition of 0.5 mL of the S-9 mix being added to overlay before pouring.

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- Place the petri dishes on a flat dry surface and allow overlay to harden. Incubate at 37°C for 72 h in an inverted position.
- Examine the background lawn of bacteria visually for proper growth. Examining the background lawn with a microscope is sometimes useful.
- Count the number of spontaneous revertant colonies and record the number. Determine whether or not the average number of colonies per plate is within an acceptable range.

Deep Rough (rfa) Character

Pipet 0.1 mL of an overnight bacterial growth (approximately 10^8 bacteria) into a 2.0-mL top agar overlay. Pour and spread the resultant mixture onto a nutrient agar plate. With sterile forceps, place a filter paper disc containing 10 µL of a 1-mg/mL solution of crystal violet in the center of the plate. Incubate for 12 to 16 h at 37°C. All tester strains should indicate the presence of the rfa mutation by a circular zone of growth inhibition (approximately 14 mm in diameter) around the disc.

Ampicillin-Resistant R-Factor Plasmid

Pipet 0.02 mL (20 µL) of an overnight broth culture (approximately 2 x 10^7 bacteria) onto a 2-mL top agar overlay. Pour and spread this mixture onto a nutrient agar plate. With sterile forceps, place a 10-µg ampicillin disc in the center of the plate. Incubate for 18 to 24 h at 37°C. Strains containing the R-factor plasmid (such as TA98 and TA100) are not inhibited by ampicillin; whereas, all other strains demonstrate a zone of growth inhibition (approximately 2-24 mm in diameter) around the disc.

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Excision Repair System (uvr B)

Pipet 0.05 mL (50 µL) of an overnight broth culture (approximately 5×10^7 bacteria) onto a nutrient agar plate. Using bent glass tubing (a "hockey stick"), spread the bacteria over the agar surface. Irradiate half of the plate at 58 cm under the germicidal lamp within a Bioguard hood for 10 s for strains not carrying the R-factor plasmid and for 20 s for strains with the R-factor plasmid.

An optional procedure is to irradiate half of the plate under a 15-watt germicidal lamp at 33 cm for 8 s. In both procedures, incubate for 18 to 24 h at 37°C. Growth should occur only on the unirradiated portion of the plate.

USE OF MASTER PLATES

To avoid continued reuse of permanent stocks and/or the creation of large numbers of frozen aliquots, master plates may be prepared and used for 1 month. The following method is suggested:

- Thaw frozen broth culture. Using a needle and syringe, add 1 mL of the broth culture to 20 mL of the nutrient broth and incubate with constant shaking (80-100 rpm) at 37°C for 16 h. Inoculate the nutrient agar master plates with 0.05 mL of the broth culture.
- Master plates will be nutrient agar plates.
- Streak each plate with the appropriate tester strain. Label plates appropriately.
- Incubate plates at 37°C for 48 h.
- Use the remaining broth cultures to perform tester strain function.

- After 48 h, store the plates at 4°C in the dark. It is advantageous to seal the plates with tape in order to prevent drying and/or to facilitate streaking from the master plates within a cold room.

- When tests are performed, a single isolated colony should be used to inoculate the overnight broth culture. This prevents a mixed population growth.

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SECTION 5

THE PLATE INCORPORATION TEST

DESIGN OF THE EXPERIMENT

Each routine test must consider the following parameters: (1) The amount of sample available for test, (2) the number of tester strains to be used, (3) performance of the test with and/or without exogenous activation, (4) spontaneous and positive controls, (5) the doses of the sample to be used, and (6) the number of replicate plates per dose. Table 2-2 provides some examples of testing routines with an estimation of the sample amount and number of plates needed.

Although complete testing cannot be done without the use of each indicator strain both with and without activation, sample size may limit the amount of testing possible. By setting a priority on the strains to be used, one can increase the likelihood of detecting active compounds. Priority assigned to the strains depends upon the type of chemical or substance to be tested. Structure-activity and source-activity relationships can be used to guide the researcher. In general, these sample size constraints and other constraints determine the number of strains to be used.

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TABLE 2-2. PLANNING SCHEME FOR SALMONELLA PLATE INCORPORATION TEST

Dose (µl/plate) per sample										
500	3	2	1	1	-	-	-	-	-	-
300	3	2	1	3	3	2	1	1	-	-
100	3	2	1	3	3	2	1	3	3	3
50	3	2	1	3	3	2	1	3	3	3
30	3	2	1	3	3	2	1	3	3	3
10	3	2	1	3	3	2	1	3	3	3
	mL (# plates)									
+ OR MA 1 strain:	3.0 (18)	2.0 (12)	1.0 (16)	2.0 (16)	1.5 (15)	1.0 (10)	0.5 (5)	0.9 (13)	0.6 (12)	0.4 (8)
2 strains:	6.0 (36)	4.0 (24)	2.0 (12)	4.0 (32)	3.0 (30)	2.0 (20)	1.0 (10)	1.8 (26)	1.2 (24)	0.8 (16)
3 strains:	9.0 (54)	6.0 (36)	3.0 (18)	6.0 (48)	4.5 (45)	3.0 (30)	1.5 (15)	2.7 (39)	1.8 (36)	1.2 (24)
4 strains:	12.0 (72)	8.0 (48)	4.0 (24)	8.0 (64)	6.0 (60)	4.0 (40)	2.0 (20)	3.6 (52)	2.4 (48)	1.6 (32)
5 strains:	15.0 (90)	10.0 (60)	5.0 (30)	10.0 (80)	7.5 (75)	5.0 (50)	2.5 (25)	4.5 (68)	3.0 (60)	2.0 (40)
+ AND-MA 1 strain:	6.0 (36)	4.0 (24)	2.0 (12)	4.3 (32)	3.0 (30)	2.0 (20)	1.0 (10)	1.8 (26)	1.2 (24)	0.8 (16)
2 strains:	12.0 (72)	8.0 (48)	4.0 (24)	8.0 (64)	6.0 (60)	4.0 (40)	2.0 (20)	3.6 (52)	2.4 (48)	1.6 (32)
3 strains:	18.0 (108)	12.0 (72)	6.0 (36)	12.0 (96)	9.0 (90)	6.0 (60)	3.0 (30)	5.7 (78)	3.6 (72)	2.4 (48)
4 strains:	24.0 (144)	16.0 (96)	8.0 (48)	16.0 (128)	12.0 (120)	8.0 (80)	4.0 (40)	7.2 (104)	4.8 (96)	3.2 (64)
5 strains:	30.0 (180)	20.0 (120)	10.0 (60)	20.0 (160)	15.0 (150)	10.0 (100)	5.0 (50)	9.0 (130)	6.0 (120)	4.0 (80)

(# Samples) X (Indicated # plates for selected scheme) + (9) X (# strains) = number plates needed.
Volume of each sample needed given directly in table.
(Volume) X (concentration/volume) = amount of sample needed.

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Although exogenous activation is difficult to define, routine testing should be done both with and without a mammalian activation system.

The range of doses in which the dose-response exists consists of nearly six orders of magnitude; however, the dose range in which a substance shows a dose-responsive curve may be very narrow, especially for complex mixtures. The minimum requirements within the HERL laboratory are five doses at half-log intervals. The highest dose should demonstrate toxicity, and the lower doses should be in the spontaneous range. For application of statistical methods, the laboratory expects three plates per dose and three doses when possible.

PROTOCOL

Select one well-grown colony from the master plate for each strain to be used and inoculate properly labeled nutrient broth flasks (20 mL per flask). Incubate these flasks with constant agitation for 16 h at 37°C.

On the day of the experiment, melt 2 mL of the top agar within the 13 x 100-mm tubes, and cool the tubes to 45°C in a water bath. It is also helpful to warm plates to 37°C prior to use, or to incubate plates overnight at 37°C if the contamination of plates is a problem.

Controls

Prepare spontaneous control plates adding 0.1 mL of bacterial broth culture and a selected amount of solvent used for test substance to the overlay

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tube. Gently vortex, pour the mixture onto the plate, and spread it evenly across the plate.

For positive controls without activation, add 0.1 mL of bacterial broth culture and 100 μL of a selected concentration of control chemical in appropriate solvent to the overlay tube. Gently vortex, pour onto plate, and spread evenly across the plate. When possible, use the same solvent as is used with the test substance.

For positive controls with activation, add 0.1 mL of bacterial broth culture, 0.5 mL of the activation mixture, 100 μL of the selected concentration of control chemical in the appropriate solvent to the overlay tube. Gently vortex, pour onto plate, and spread evenly across the plate. When possible, use the same solvent as is used with the test substance.

The Test Substance

Add 0.1 mL of the bacterial broth culture. Next add the appropriate concentration of test substance. When activation is to be used, also add 0.5 mL of the activation mixture to the overlay tube. Gently vortex, pour the mixture onto the plate, and spread it evenly.

After overlays have hardened, invert and incubate in the dark at 37°C for 72 h.

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After this 72-h period, examine each plate for background growth. Then count the number of colonies per plate. If there are fewer than 20 colonies per plate, counting can be done visually; otherwise, electronic counting machines may be used.

Select at random from test substance plates colonies that are restreaked onto minimal media. Check the activation mixture and test substances by overlaying them onto VBME agar plates.

Record all results onto computer-compatible sheets; the responsible investigator should initial all sheets.

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SECTION 9

RECORDING OF RESULTS AND QUALITY CONTROL

Record all results in a permanent laboratory record book and on standard forms for computerization (see Part III). Five forms are currently available for recording data for keypunching. The "Sample Identification Form" allows the user to register an identification code and an associated description of the test substance. The identification code is constructed in the following manner:

- The code consists of three parts separated by dashes with an AAAA-XX-YYYY format.
- AAAA is the code for the source of the sample.
- XX is the code for the year of generation or procurement.
- YYYY is either the sequential or randomly-selected unique number for the sample.

After assigning an identification code, give a short description of the sample. On the "Results Form," have the laboratory technician enter information that uniquely identifies a test: a four-letter code for the research laboratory performing the test, the sample identification code, the test date, the bacterial strain used, an optional strain batch identifier, and an optional identifier for the batch of exogenous activation enzyme mixture

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when used. Next the technician should enter quality control and quality assurance information such as results of a check for phenocopy colonies, the sterility for the activation mixture and sample, the amount of activation mixture used, the time and temperature of any pre-incubation conditions, and the name of the technician performing the test. If the technician determines that additional comments are needed, he/she should place a 1 in box 64 and record his/her comments at the bottom of the results form. Following that, the technician records the counts of revertant colonies per plate noted for varying doses of test agent, as well as for the positive control and negative control. Within one test, the technician may enter a maximum of 24 doses (by using the "Results Continuation Form") with up to 5 plates at each dose level. (The design of these forms was based on those in use at the National Cancer Institute [NCI].)

To provide good quality assurance data on the activation mixtures used, use the two forms provided to record essential and useful information. The first form records information concerning the preparation of the activation system. Included is information concerning: (1) the supplier, if other than the research laboratory, (2) the animal(s) used, (3) the type of induction, if any, used, and (4) the details of preparation. The second form records any quality control information gathered. These forms are based on former work completed at the National Institute for Environmental Health Sciences (NIEHS) and previous efforts by NCI and the National Center for Toxicology Research (NCTR).

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SECTION 10

REPORTING AND ANALYSIS OF TEST RESULTS

Three report formats are currently available that list the entire data base or selected subsets. The "Plate Count Report" lists individual plate counts, mean, and standard deviation at each dose level on a test-by-test basis. Tests are grouped in order by test agent, sample code, and research laboratory performing the test. The user may select the limits of this report by specifying a research laboratory and/or ranges of sample identification codes. The "Plate Count Summary Report" groups test results for reporting on an experiment-by-experiment basis. An experiment is defined as all of the tests performed on a single day in a particular research laboratory. The user has the option of limiting the scope of this report according to research laboratory and/or experiment date. For example, he/she can easily request that only experiments performed between January 1, 1979 and January 10, 1979 by laboratory XXXX be included in the report. The "Statistical Summary" page displays the raw data for a test and various statistics pertaining to the test results. Next to this information appears a graph of plate counts versus test agent dose level. The curved line is generated by fitting a linear model to the observed plate counts.

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The "Plate Count Report" and the "Plate Count Summary Report" are designed for output to a CRT screen and an attached hard-copy unit. Data from the "Statistical Summary" also can be output via a standard line printer. Requests for reports and specifications of data selection criteria can be done from an inactive terminal if desired.

Experimental data selected by this program can also be input to another program, GRAPH60, which generates CRT graphs that display dose versus mean plate count for multiple microorganism strains or test samples within an experiment.

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APPENDIX I

Laboratory: Genex Laboratories
12300 Washington Ave.
Rockville, MD 20852

Submitter: Dr. Vincent F. Simmon
General Manager
Genetic Toxicology Division
(301)468-6622

Date of submission: February 17, 1981

Omissions: None

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Genex

Reply to:

Genex Laboratories
12300 Washington Ave.
Rockville, Md. 20852
(301) 468-6622

February 17, 1981

Ms. Stephanie Toney
USEPA, HERL, GTD (MD-68)
Research Triangle Park, NC 27711

Dear Ms. Toney:

Enclosed is our Ames Test Protocol for the standard plate incorporation assay. We have slightly different protocols for preincubation, desiccator, and liquid suspension assays.

I'm sorry for the delay in sending this protocol to you.

Sincerely,

Vincent F. Simmon

Vincent F. Simmon, Ph.D.
General Manager
Genetic Toxicology Division

VFS:ljh

Enclosure

Genex Corporation · 6110 Executive Blvd. · Rockville, Md., 20852 · 301-770-0650

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Ames Salmonella/microsome Assay

Standard Plate Incorporation Protocol

1. Background

The *Salmonella typhimurium* strains (TA1535, TA1537, TA1538, TA98, and TA100) used in this assay require histidine for growth. The assay measures the reverse mutation of the strains to histidine independence when exposed to test samples.

To duplicate some aspects of mammalian metabolism, an homogenate of rat liver and a mix of cofactors are included in the assay.

2. Bacterial Strain Maintenance and Inoculation

The *S. typhimurium* strains are kept at approximately 4°C on minimal medium plates (1.5% agar, 1% glucose, 0.02% $MgSO_4 \cdot 7H_2O$, 0.2% citric acid monohydrate, 1% K_2HPO_4, and 0.35% $NaNH_4PO_4 \cdot 4H_2O$) supplemented with an excess of histidine and biotin. The plasmid-carrying strains are kept on plates containing 25 ug/ml ampicillin to ensure stable maintenance of the plasmid. New stock culture plates are made every month from frozen master stocks and are checked for their genotypic characteristics (*his*, *rfa*, *bio*), spontaneous mutation frequency, and for the presence of the pKM101 plasmid. For each experiment, an inoculum from the stock culture plates is grown with aeration overnignt at 37°C in nutrient broth (Oxoid No. 2, CM67).

3. Plate Incorporation Assay

To a sterile 13 x 100 mm test tube we add, in the following order,

a) 2.0 ml top agar (0.6% agar, 0.05 mM histidine, 0.05 mM biotin)
b) 0.05 ml overnight culture of bacteria
c) 0.05 ml test sample dilution, or positive or negative control
d) 0.5 ml S-9 Mix (optional)

The contents are vortexed and poured onto minimal medium plates. After the top agar has set, the plates are incubated at 37°C for three days. The number of *his^+* revertant colonies (large visible colonies) is counted and recorded. Colonies are counted using a BioTran II Automated Colony Counter when possible. If not possible, colonies are counted using the electric probe colony counter.

Each sample will be tested in a preliminary experiment (one plate per dose) followed by two full experiments in duplicate.

4. Dosage Selection

Eight doses are tested with and without S-9 Mix, using strain TA100 only, in the preliminary experiment. The top dose will be 10 ul/plate, or the maximum soluble dose. The remaining doses will be serial half-log dilutions of the top dose. Five doses to be tested in subsequent experiments are selected based on toxicity, mutagenicity, and or solubility observed in the preliminary experiment. Toxicity is indicated by a reduction of the number of spontaneous revertants, a clearing of the background lawn, or the appearance of his^- pinpoint colonies.

5. Solvent

The solvent used is that recommended by the client. If no solvent is recommended, the test sample will be examined for solubility in dimethylsulfoxide (DMSO). If the sample is not soluble in DMSO, a solvent will be selected that is mutually acceptable to the Principal Investigator and the Study Director.

6. Controls

Positive and solvent controls will be included in each experiment.

Positive controls	Dose (ug/plate)	Strain	S-9 Mix
2-aminoanthracene (2-anthramine)	2.5	all five	+/-
sodium azide	1.0	TA1535,TA100	-
9-aminoacridine	50.0	TA1537	-
2-nitrofluorene	5.0	TA1538, TA98	-

Crystal violet control

To verify that the overnight cultures of the tester strains have retained the rfa characteristic, a loopful of the culture of each strain is streaked on a nutrient plate. When the streaks are dry, a filter disc (6.4 mm diameter) is placed on each streak and 10 ul of 2% crystal violet is pipetted onto the disc. This plate is incubated overnight at 37°C and the zone of inhibition of growth is measured and recorded.

7. S-9 Mix Preparation

Some carcinogenic chemicals are inactive unless they are metabolized to active forms. Enzymes in the liver and in other organs are capable of metabolizing a large number of chemicals to carcinogens. Therefore, we include an homogenate of rat liver in this assay.

Adult male Fischer 344 rats (200 to 250 g) are given a single 500 mg/kg intraperitoneal injection of Aroclor 1254® in corn oil. This treatment enhances the synthesis of enzymes involved in the metabolic conversion of chemicals. Four days after the injection, the animals' food is removed but drinking water is provided *ad libitum*. On the fifth day, the animals are sacrificed with a stainless steel guillotine. The livers are removed aseptically and the liver homogenate is prepared as follows:

The livers are placed in a preweighed sterile glass beaker and their total weight is determined. All procedures are conducted in an ice bath. The livers are washed in an equal volume of cold, sterile 0.15 M KCl (1 ml per g of wet organ), minced with sterile surgical scissors in three volumes of 0.15 M KCl, and homogenized with a Potter-Elvehjem apparatus. The homogenate is centrifuged for 10 minutes at 9000 x *g*, and the supernatant (referred to as the S-9 Fraction) is quickly frozen in dry ice and stored at -80°C.

The S-9 Mix is made up fresh for each experiment and is kept in an ice bath. The S-9 Mix consists of, for 10 ml:

1.0 ml	of S-9 Fraction
24.6 mg	of KCl
16.2 mg	of $MgCl_2$
14.0 mg	of glucose-6-phosphate
31.5 mg	of NADP
5.0 ml	of sodium phosphate buffer (0.2 M, pH 7.4)
3.35 ml	of distilled H_2O

8. Interpretation

A test sample is considered mutagenic if it induces a reproducible, dose-related increase in *his*$^+$ revertants in at least one of the tester strains. A test sample is considered not mutagenic if there are no dose-related increases in revertants and if it is tested over a broad range of doses up to a toxic dose. If test results are equivocal or controls are not acceptable, an additional experiment(s) is conducted.

APPENDIX J

Laboratory: Hazleton Laboratories America, Inc.
9200 Leesburg Tnpk.
Vienna, VA 22180

Submitter: Nancy E. McCarroll
Staff Scientist
Mutagenicity Section
Department of Toxicology
(703)893-5400

Date of submission: December 3, 1980

Omissions: None

HAZLETON
LABORATORIES AMERICA, INC.
9200 LEESBURG TURNPIKE VIENNA VIRGINIA 22180 U S A

December 3, 1980

Ms. Stephanie Toney
USEPA, HERL, GTD, GBB (MD-68)
Research Triangle Park, NC 27711

Dear Ms. Toney:

Enclosed is Hazleton's standard protocol for an Ames Test. As you probably realize, it is many times necessary to make minor modifications in this procedure for specific test compounds.

I hope this is of use to you. Please send a final copy of the compiled protocols.

Sincerely,

NANCY E. McCARROLL
Staff Scientist
Mutagenicity Section
Department of Toxicology

NEM/pn
Enclosure

PHONE (703) 893 5400 TELEX 899436 (HAZLABS VINA) CABLE HAZLABS WASH DC

HAZLETON
LABORATORIES AMERICA, INC.

PROTOCOL

1. Study — *Salmonella typhimurium* mammalian microsome plate incorporation assay.

2. Purpose — To evaluate the mutagenic potential of Compound ______________________ as determined by the induction of reverse mutations using five strains of *S. typhimurium* in the presence and absence of metabolic activation.

3. Justification for Selection of the Test System

The *Salmonella*/Mammalian-Microsome Mutagenicity Assay of Ames is a rapid, reliable, and economical method of screening compounds for potential genetic activity at the single base pair level. A large data base has been accumulated with this assay, confirming its ability to detect genetically active compounds at an efficiency of about 80%.

The In Vitro Test System Species: *Salmonella typhimurium*

STRAINS: TA1535, TA1537, TA1538, TA98, TA100 (de Serres, F.J. and M. D. Shelby, *Environ. Mutagenesis* 1 (1979) 87-92). These histidine-requiring strains are characterized by deletions through the gal-bio-uvrB region and require histidine as well as biotin for growth. The specific mutations for each strain are shown in the following table.

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Strain	Contains Mutations To histidine biosynthesis	excision repair	cell wall	Contains error-prone repair system
TA1535	base-pair substitution	yes	yes	no
TA1537	frameshift	yes	yes	no
TA1538	frameshift	yes	yes	no
TA98	frameshift	yes	yes	yes
TA100	base-pair substitution	yes	yes	yes

When exposed to a mutagen, some of the bacteria, through interaction with the mutagen, undergo genetic changes which cause them to revert to a non-histidine-deficient state and thus grow and form colonies in the absence of exogenous histidine. The number of colonies which form after exposure to a mutagen is directly related to the potency and concentration of that particular mutagenic compound, and the type of mutations the compound produces. The rate of spontaneous reversion to histidine prototrophy and the responsiveness and sensitivity of the strains to mutagenic action are determined in conjunction with each test.

4. Test Material

   A. Identification

   B. Purity

   C. Method of synthesis, stability, and composition

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D. Other

5. Experimental Design

The standard assay will be routinely performed with five S. typhimurium tester strains at a minimum of five test compound doses per strain both with and without metabolic activation by an Aroclor-induced rat liver microsomal fraction. The test compound dose levels will be determined by a preliminary multi-dose-ranging (toxicity) study. Appropriate negative (solvent) and positive controls will be included with each assay. All doses will be plated in triplicate.

A. Integrity of Test System

(1) Tester strains are maintained frozen and fresh broth cultures will be prepared daily for each experiment from these stocks.

(2) Spontaneous revertant frequencies will be determined and must be within acceptable limits for each study. (See de Serres, F.J., and M.D. Shelby, Mut. Res., 64 (1979) 159-165 for typical spontaneous reversion frequencies.)

(3) The presence of the specific genetic markers will be determined periodically for each tester strain. Each organism will be routinely checked for:

(a) histidine requirement.

(b) biotin requirement (for uvrB deletion).

(c) sensitivity to crystal violet and/or deoxycholate (for deep rough (rfa) mutation to cell wall).

(d) ampicillin resistant R factor (for tester strains TA100 and TA98). (See de Serres, F.J., and M.D. Shelby Science 203 (1979) 563-565 for details).

HAZLETON
LABORATORIES AMERICA, INC.

(4) Each new batch of S-9 will be checked with standard mutagens to evaluate its strength and to find the optimum amount to use for general screening.

(5) To ensure uniformity throughout the assay, the medium will be prepared in bulk volumes and then aseptically dispensed into culture plates.

(6) All doses will be plated in triplicate.

B. Solubilization of Test Material

C. Toxicity Evaluation

The greatest number of induced mutations per plate and therefore the most sensitive test for mutagenesis should result from treatment of the indicator organisms with the highest concentration of chemical that causes minimal killing of the organisms. The exact kinetics of toxicity and mutation induction will vary with different compounds; however, in general, the optimal concentration should allow survival of about 50 percent of the cells. The method described below will be used in our laboratory to indicate the levels at which compounds should be tested for mutagenesis.

The concentration of the chemical found satisfactory for testing based on the solubility study will serve as the initial dose for the toxicity evaluation. A minimum of five tenfold serial dilutions of the agent will be prepared in the appropriate solvent to contain the test dose in 0.1 ml volumes. In general, strain TA100 has proven to be the most resistant strain to chemical toxicity and is used as the indicator in the absence of a liver microsomal preparation for routine toxicity determination in our laboratory.

To each of 2 ml of complete top agar, 0.1 ml of an overnight broth culture of S. typhimurium strain TA100 and 0.1 ml of the appropriate dilution of the test compound or diluent will be added. The contents of the tube will be thoroughly mixed, poured and uniformly distributed over VBE minimal agar. The plates will be allowed to harden approximately one hour, inverted and put into a dark 37± 0.5°C incubator. After two days, the background lawn of growth and revertant colonies in both test and control plates will be scored. Observations of chemical precipitation will be similarly recorded. Inhibition of the bacteria as indicated by a reduction in the bacterial lawn and/or a decrease in spontaneous revertants will be recorded at each concentration. This test will provide the data used for selecting the doses to be used in the mutagenicity assay. If toxicity is not evident at any of the doses, higher concentrations of the compound will be examined. The upper dose limits will be determined by the extent of chemical precipitation which interferes with the observation of growth.

D. Mutagenicity Assay

(1) Design and Dosing

Exposure of auxotrophic bacteria will be done directly by the plate incorporation assay. For the induction of reverse mutations, these chemicals will be tested against the five bacterial strains with and without the metabolic activation systems. Each concentration of the compound and controls will be plated in triplicate.

To ensure uniformity in all experiments, media will be prepared in bulk volumes. Twenty-ml aliquots will be aseptically dispensed, using a Petrimat, into 100x15mm disposable petri dishes and stored at room temperature in the same location until required. All other media and reagents will be prepared in a similar manner.

Data derived from the toxicity studies will be utilized to establish test levels of each compound for further investigation. A minimum of five concentrations of the test compound will be prepared to contain the test dose in 0.1 ml volumes for evaluation with and without metabolic activation. The highest dose tested will be that level of the chemical exhibiting inhibition of the bacterial growth in toxicity studies, unless this is not possible due to limited solubility of the investigated agent.

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To each of 2 ml of complete top agar, 0.1 ml of an overnight broth culture of each tester strain, 0.1 ml of the test compound or diluent, and 0.5 ml of the S-9 mix for the activated tests will be added. The contents of the tube will be mixed thoroughly and poured onto VBE minimal agar plates. Plates will be gently rotated and tilted to assure uniform distribution of the top agar, allowed to harden on an even surface for one hour, inverted and put in a dark 37± 0.5°C incubator. After two days, the colonies in both test plates and controls will be counted using an electronic colony counter and the density of the background growth will be noted.

(2) Preparation of Chemical Solutions

All concentrations will be on a weight per volume basis; and the compounds will be dissolved in an appropriate solvent.

(3) Control Compounds

(a) Negative Control(s)

The solvent used for the test compound will be used as the negative control(s) at the same concentration(s) as used with the test compound.

(b) Positive control compounds (appropriate for each metabolic activation condition) will be included in the final experiment. They were chosen to reflect the responsiveness of the tester strains as well as the integrity of the test system especially in conjunction with metabolic activation. The positive control compounds used with this assay are:

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HAZLETON
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Strain	Minus S-9	Plus S-9
TA1535	MNNG (5µg/plate)	2-AA (5µg/plate)
TA1537	9-AA (75µg/plate)	2-AA (5µg/plate)
TA1538	2-NF (50µg/plate)	2-AA (5µg/plate)
TA98	2-NF (50µg/plate)	2-AA (5µg/plate)
TA100	MNNG (5µg/plate)	2-AA (5µg/plate)

Abbreviations: MNNG = N-Methyl-N'-nitro-N-nitrosoguanidine

9-AA = 9-Aminoacridine

2-NF = 2-Nitrofluorene

2-AA = 2-Aminoanthracene

(4) Codes used for Mutagenicity Assay without metabolic activation

(a) Each plate is labeled with a six digit number.

(b) The first two numbers refer to the bacterial strain:

01 = 1535
02 = 1537
03 = 1538
04 = TA98
05 = TA100

(c) Numbers in the third and fourth positions refer to the compound.

(d) Numbers in fifth and sixth positions refer to the dilution of the test compound.

(e) Controls

Negative

ORG = 01-050000 media control (expected spontaneous reversion)

DMSO = 01-050001

Compound Solvent = 01-050003 if compound solvent is different than positive control solvent.

Positive

01 and 050004 = 5 μg/plate MNNG with 1535 and TA100

020004 = 75 μg/plate 9-AA with 1537

03 and 040004 = 50 μg/plate 2-NF with 1538 and TA98

(5) Code used for mutagenicity assay with metabolic activation

Identical to that used for the mutagenicity assay without activation with the following exceptions:

(a) Bacterial strains are coded as double digit numbers

11 = 1535
22 = 1537
33 = 1538
44 = TA98
55 = TA100

(b) Positive Control

11-550004 = 5 μg/plate 2-AA tested with all bacterial strains.

(6) Metabolic Activation

The mammalian microsome fraction used for metabolic activation will be prepared from male Sprague Dawley rats. The liver homogenate (S-9) will be prepared in advance and stored frozen until used.

(a) Preparation of Liver Homogenate (S-9)

Enzymes will be induced in male Sprague Dawley rats by a single intraperitoneal injection of polychlorinated biphenyl mixture (200 mg Monsanto Aroclor 1254/ml corn oil) at a dosage of 500 mg/kg five days before sacrifice. The animals will be deprived of food and water prior to sacrifice.

The rats will be sacrificed by decapitation, the livers removed, placed in sterile ice-cold beakers, and washed in an equal volume of 0.15M KCl. All livers will be minced with sterile scissors in three volumes of the sterile KCl rinsing solution and homogenized. The homogenates will be centrifuged at 9000xg in a refrigerated centrifuge and the supernate (S-9 fraction) retained. The S-9 fraction will be distributed in aliquots quickly frozen on dry ice, and stored in a freezer. As needed, sufficient S-9 fraction will be thawed and kept on ice until incorporated into the S-9 mix. The unused portion will be discarded at the end of the day.

(b) Preparation of S-9 Mix

The S-9 mix contains per ml:

a.	S-9		.05* ml
b.	$MgCl_2$	0.4M	.02 ml
c.	KCl	1.65M	.02 ml
d.	NADP	0.05M	.08 ml
e.	glucose-6-phosphate	0.1M	.05 ml
f.	Sodium phosphate pH 7.4	0.2M	.5 ml
g.	H_2O		.28 ml
			1.00 ml

*The appropriate amount of S-9 to be added will be determined for each batch of liver homogenate on the basis of standardization experiments for the particular batch using the promutagens 2-acetylaminofluorene and 2-aminoanthracene.

The S-9 mix will be freshly prepared on the day of the experiment. Ingredients a, b, c, f, and g will be mixed, passed through Millipore prefilter, 0.8µ membrane and 0.45µ sterile membrane. Ingredients d and e will be aseptically added following filtration. The S-9 mix will be kept on ice; unused portion will be discarded at the end of the day. Stock solutions of $MgCl_2$, KCl, NaH_2PO_4 will be stored in the refrigerator and stocks of reagents G-6-PO_4 and NADP will be held in a freezer.

(7) Results and Interpretation

S. typhimurium revertants to histidine prototrophy on test and control plates will be counted with an electronic colony counter and the number of revertants on treated and control plates will be compared. While the results of each experiment will be considered independently, the spontaneous reversion rate and the sensitivity and reproducibility of the bacterial strains and metabolic activation systems will be evaluated both by reference to previous work and by the concurrent use of positive and negative controls.

The criteria outlined below have been established in our laboratory for evaluation of Ames Assay.

For a test to be considered valid, the following conditions are required:

1. Demonstration of toxicity of the chemical for the *S. typhimurium* strain(s), unless this is not possible due to limited solubility of the test compound.
2. The negative control responses are within the normal range of our laboratory data base.
3. Confirmation of sensitivity and responsiveness of the tester strains to mutagenic action as indicated by their responses to the positive controls.

If the above criteria are met, a chemical is considered to be mutagenic if it induces a positive response in a dose-related manner over three concentrations with the baseline increase in the number of histidine revertants equal to twice the solvent control level.

(8) Reports

A. Interim Report(s)

If requested, interim oral (telephone) report(s) will be made to the sponsor's designated representative.

B. Final Report

At the termination of the study a final report which includes the following information will be submitted:

Experimental objectives, design and methods

Results

Toxicity evaluation

Test results

1. Presentation of the number of revertants obtained from the mutagenesis assay for each strain, condition of metabolic activation and dose, including negative and positive controls.
2. Processed raw data including tables, graphs, conversions and statistical analyses, where applicable.

Interpretation of the Results

Conclusions

(9) Data Storage

Data generated during the course of the study will be maintained in Room 2348, Building 19.

At the completion of this study, the raw data and final report will be sent to the Archives of Hazleton Laboratories America, Inc.

APPENDIX K

Laboratory: Hill Top Research, Inc.
P.O. Box 42501
Cincinnati, OH 45242

Submitter: Dr. George C. Lavelle
Director of Technical Services
(513)831-3114

Date of submission: December 23, 1980

Omissions: None

Hill Top Research, Inc.
Miamiville, Ohio 45147 (513) 831-3114

December 23, 1980

Stephanie Toney
Genetic Bioassay Branch (MD 68)
United States Environmental
Protection Agency
Health Effects Research Laboratory
Research Triangle Park
North Carolina 27711

Dear Ms. Toney,

In response to your letter of November 26, 1980, addressed to Mr. Ward Billhimer, please find enclosed a copy of our standard Ames protocol (Plate Incorporation Assay). I would like to have a copy of the final compilation.

Please note that our Genetic Toxicology Section is now part of the Department of Toxicology at Hill Top Research, Inc.

Sincerely yours,

GC Lavelle

George C. Lavelle, Ph.D.
Director of Technical Services

GCL/lc

Enclosure

Hill Top Research, Inc.

P.O. Box 42501, Cincinnati, Ohio 45242 (513) 831-3114

PROTOCOL
GENE MUTATION STUDY

Salmonella/Mammalian Microsome Assay (Ames)
Plate Incorporation Test

PURPOSE

The Salmonella/Microsomal assay is designed to provide an evaluation of the mutagenic potential of chemicals using strains of *Salmonella typhimurium* (Ames) as indicator organisms (Ames *et al*, 1975).

TESTING FACILITY

Hill Top Research, Inc.
Miamiville, Ohio 45147 (513) 831-3114

PROPOSED STARTING DATE

One (1) to two (2) weeks after receipt of the test material(s) and approved study protocol.

PROPOSED COMPLETION DATE

Verbal results will be given at completion of study with written report to follow within one month of completion.

TEST SYSTEM JUSTIFICATION

The Ames Test, a short-term bacterial assay used to detect point mutations, has been designated a Tier 1 *in vitro* assay in mutagenicity testing. The plate incorporation test is an accepted method for quantitative dose-response assay of water - or hydrocarbon - soluble materials.

Mutagenicity Study (Ames)

TEST ORGANISMS

Salmonella typhimurium strains TA98, TA100, TA1535, TA1537, and TA1538 will be used. All strains were received from Dr. Bruce N. Ames, University of California, Berkeley. Growth medium will be nutrient both. Permanent stock seed cultures are stored at -80 C. Tester strains are regularly monitored for genetic markers, spontaneous reversion, and mutagenesis with standard mutagens.

TEST ARTICLE PREPARATION

Water soluble test materials will be prepared in water. Non-water soluble materials will be prepared in dimethysulfoxide.

REAGENTS

1. Media

Minimal-Glucose Mutation Agar Medium

$MgSO_4$ $7H_2O$	0.2 gm
Citric Acid H_2O	5.0 gm
K_2HPO_4	10.0 gm
$NaNH_4PO_4$	3.5 gm
NaCl	5.0 gm
Agar	15.0 gm
H_2O	1000.0 ml

The media will be autoclaved and supplemented with two percent (2%) glucose prior to pouring plates.

Top Agar Medium

Agar	6.0 gm
NaCl	5.0 gm
H_2O	1000.0 ml

Top agar will be sterilized by autoclaving and supplemented with 10.0 ug histidine HCl and 12.0 ug biotin per ml of media.

Nutrient Broth

Sterile nutrient broth will be used for overnight growth of indicator organisms.

Tryptone Agar Medium

Tryptone	10.0 gm
Agar	15.0 gm
H_2O	1000.0 ml

2. Microsomal Supernatant Fraction (S-9)

Induction of Rat Liver Enzymes

Sprague Dawley male rats (approximately 200 grams each) will be used for the induction of liver enzymes. A single intraperitoneal injection of chlorinated biphenyls (Arochlor 1254, diluted in corn oil to a concentration of 200 mg/ml) at a dosage of 500 mg/kg is given to each rat five (5) days before sacrifice. The rats will be given drinking water and feed ad-libitum until 12 hours before sacrifice, at which time the food will be removed. On the fifth day of induction, rats are stunned and then decapitated.

Preparation of Liver Homogenate

All steps will be performed at 0 - 4 C using ice baths, sterile solutions and glassware. The livers are placed in pre-weighed beakers containing 0.15 M KCl. After being weighed, livers are transferred to a beaker containing 3 ml of 0.15 M KCl per gram of wet liver. Livers are minced with sterile scissors and homogenized in a Potter-Elvehjem apparatus with a Teflon pestle. The homogenate is centrifuged for 10 minutes at 9000 x g and the supernatant (S-9) is decanted and saved. Protein contents and sterility will be determined for each batch of S-9 prepared. The S-9 fraction will then be dated, quick frozen and stored at -80 C.

Preparation of Working S-9 Mix

The S-9 mix will contain per milliliter: 4 umoles NADP (nicotinamide adenine dinucleotide phosphate), 5 umoles G-6-P (glucose-6-phosphate), 8 umoles $MgCl_2$, 33 umoles KCl, 100 umoles sodium phosphate (pH 7.4). The level(s) of S-9 will be determined by the activity of the S-9 batch used.

Mutagenicity Study (Ames)

CONTROLS

Positive Controls

Non-activation

N-methyl-N-nitro-N-nitrosoguanidien (TA100, TA1535)
2-Nitrofluorene (TA98, TA1538)
9-Aminoacridine (TA1537)

S-9 Activation

2-Anthramine (all strains)

Negative Controls

Both a solvent and a non-solvent negative control will be included.

PROCEDURES

1. Toxicity Determination

The effect of the test article(s) on the survival of the bacterial strains will be determined prior to the Ames Bioassay. This will be accomplished by adding different levels of the test article(s) and solvent(s) to tubes contaning 2.0 ml top agar (at 45 C) and 0.1 ml of the tester strain. After mixing, the tube contents will be poured onto the surface of tryptone agar plates. The plates will then be incubated at 37 C for 18-24 hours and the background lawn of bacteria in test article(s) plates will be compared to the bacterial lawn in the solvent(s) plates. Toxicity on the tryptone agar plates will be detectable by a thinning or disappearance of this back-ground lawn of bacteria.

2. Plate Incorporation Test

Non-activation assay

S. typhimurium TA98, TA100, TA1535, TA1537, and TA1538 will be used.

Mutagenicity Study (Ames)

Five dose levels of the test article(s) will be employed. These levels will be based on the results of the toxicity determinations. The highest dose level will be one which demonstrates toxicity.

This test will be conducted by adding 0.1 ml of each dose level of the test article(s) to tubes containing 2.0 ml of top agar (at 45 C) and 0.1 ml of a 24-hour nutrient broth culture of the tester strains. The tube contents will be mixed and poured onto the surface of minimal-glucose agar plates. All plates will then be incubated at 37 C for 48-56 hours. After incubation, the number of revertants will be counted and compared with the number of revertants on negative and positive control plates.

Results will include a mutagenic activity ratio for each dose level of the test article(s) and positive controls. This ratio will represent the average number of revertants per dose level of test article(s) or positive controls divided by the average number of revertants in the negative control.

Activation Assay

This test will be conducted as described above (Non-activation assay) with the addition of 0.5 ml of S-9 mix to the tubes prior to pouring the contents onto minimal-glucose agar plates.

Results will be reported as above in the non-activation plate incorporation assay.

EVALUATION

Evaluation of the results shall include a determination of the dose-response relationship, if any, between the levels of the test article and the number of revertants recorded for each level.

REPORT

The final report will include (but will not be limited to) a description of the test procedure, description of the test material (including date of receipt, color, form and any obvious odors), concentration of sample, solvents, dosage level of test and control materials, and batch of S-9 mix.

Mutagenicity Study (Ames)

The report will also include the experimental design and the mean, standard deviation and mutagenic activity ratio in tabular form of the number of revertants for each dose level of negative controls, positive controls and test articles assayed by the quantitative plate incorporation method.

NOTICE

This study will be run according to Good Laboratory Practices regulations. If it becomes necessary to make changes in the approved protocol, the revisions and reasons for change will be documented, reported to the sponsor and will become part of the permanent file for that study. Similarly, the sponsor will be notified as soon as is practical whenever an event occurs that is unexpected and may have an effect on the validity of the study.

DATA RETENTION

The raw data and the original of the final report will be on file at the testing facility for a period of not less than two years. The sponsor will be notified before final disposition of these items. Unused test material will be destroyed unless indicated otherwise by the sponsor.

REFERENCES

Ames, B. N., J. McCann and E. Yamasaki, Mutation Research 31, 347-364 (1975).

PROTOCOL APPROVED BY:

Mark R. Entrup, B.S. Section Head, Genetic Toxicology	Date	George C. Lavelle, Ph.D. Director of Technical Services, Genetic Toxicology	Date

PROTOCOL REVIEWED AND ACCEPTED:

By: ______________________ Date: ______________
Sponsor

By: ______________________ Date: ______________
For

SUPPLEMENTAL INFORMATION FOR GENETIC TOXICOLOGY
PROTOCOL NO. ____________________ DATED __________

REGULATORY AGENCY

This study will be implemented in conformance with Good Laboratory Practice regulations (21 CFR 58) for nonclinical studies. The study (is) (is not) intended for submission to a regulatory agency. Name of agency(ies):

____

____

TEST AND CONTROL ARTICLES

All test articles are furnished by the sponsor. They are identified as follows: ____

____

The sponsor assumes responsibility for any chemical evaluations for purity, strength, stability, etc.

The identity, strength, uniformity, composition, purity of the test and/or control substances have been determined and documented as of __________.
(Date)

The method of synthesis or origin of the test and control substances, including their amount and the method of assay have been documented:
Yes ______ No ______

The stability of the test and/or control substances have been determined or will be determined as of ________________________.
(Date)

Storage conditions: ____

Handling precautions: ____

SUPPLEMENTAL INFORMATION

OTHER PERTINENT INFORMATION

PROTOCOL MODIFICATIONS

SPONSOR AND ADDRESS

Authorized Signature Date

Position

HTR/TD/11-80

HILL TOP RESEARCH ABSTRACT PROTOCOL

Salmonella/Mammalian Microsome Assay (Ames)
Plate Incorporation Test

PURPOSE

The Ames Plate Incorporation Test is designed to provide an evaluation of the mutagenic potential of chemicals using strains of *Salmonella typhimurium* as indicator organisms.

TEST SPECIES

Salmonella typhimurium strains TA 98, TA 100, TA 1535, TA 1537, and TA 1538.

SAMPLE REQUIRED

One (1) gram of sample needed.

DURATION OF TEST

Four weeks are required from the day of proposed start until proposed completion and report out to sponsor.

TEST PROCEDURE

The quantitative plate incorporation assay uses solutions of the test material at five levels with five strains of *S. typhimurium*, with and without metabolic activation provided by microsomal rat liver homogenate.

OBSERVATIONS

For quantitative assays, numbers of revertant colonies are enumerated and compared with revertant colonies in negative and positive controls. Results of toxicity tests are included.

REPORT

Final report will contain the experimental design, the results in tabular form with statistical analyses, and conclusions as to the mutagenic potential of the test material.

NOTICE

This study will be run according to 21 CFR 58 (Good Laboratory Practices regulations) and 40 CFR 163 (EPA).

REFERENCE CODE

5-1-1/11-1-80/REV-1

APPENDIX L

Laboratory: IIT Research Institute
10 W. 35th St.
Chicago, IL 60616

Submitter: Peter W. Barbera
Associate Microbiologist
Life Sciences Research
(312)567-4000

Date of submission: December 4, 1980

Omissions: None

IIT Research Institute
10 West 35 Street Chicago Illinois 60616
312 567-4000

December 4, 1980

Dr. Stephanie Toney
USEPA, HERL, GTD, GBB (MD-68)
Research Triangle Park
North Carolina 27711

Dear Dr. Toney:

Your letter of November 26 was referred to me by Dr. James Fenters. As you requested we are inclosing a copy of our Ames *Salmonella* Mutagenicity protocol for use in your Ames test compilation.

Sincerely,

Peter W. Barbera

Peter W. Barbera
Associate Microbologist
Life Sciences Research

encl.

PWB/rmk

AMES SALMONELLA/MAMMALIAN-MICROSOME TESTS FOR DETECTION OF CARCINOGENS AND MUTAGENS

- Plate Incorporation Test
- Spot Test

Life Sciences Research
IIT Research Institute
10 West 35th Street
Chicago, Illinois 60616

May 1980

IIT RESEARCH INSTITUTE

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AMES SALMONELLA/MAMMALIAN-MICROSOME TESTS FOR DETECTION OF CARCINOGENS AND MUTAGENS

I. INTRODUCTION

The Ames test is a simple, reproducible *in vitro* test to detect potential carcinogens by their ability to cause base-pair and frame-shift mutations in bacterial DNA. The mutagenic events are actually reverse mutations in that they cause histidine-requiring mutants to revert to the prototrophic (non-histidine-requiring) state. Special strains of *Salmonella* are used to measure DNA damage, combined with tissue homogenates from the rat to provide mammalian metabolism of chemicals. The chemical to be tested, approximately 10^9 bacteria of a particular tester strain (five histidine-requiring-mutants are used), and rat liver homogenates are combined on a dish and after incubation at 37°C for 2 days histidine revertants are scored. Ames has found an 85 to 90% correlation between carcinogenicity and mutagenicity (Ames et al., Mut. Res. 31, 347-364, 1975). Ames also states that the Aroclor 1254-induced rat liver enzymes result in the most efficient detection of different classes of compounds.

II. MATERIALS AND METHODS

A. Salmonella Strains

Five mutant strains of *Salmonella typhimurium*, TA98, TA100, TA1535, TA1537 and TA1538 received from Dr. Bruce Ames at the University of California, Berkeley are used in our laboratories. All strains have a rfa (deep rough) mutation that eliminates the polysaccharide side chain of the lipopolysaccharide layer coating the bacterial surface, thereby making the bacteria more permeable to test chemicals. All strains also lack the DNA repair system, which greatly increases sensitivity to mutagens. TA98 and TA100 contain an ampicillin-resistant R factor that makes them more sensitive to chemicals, mainly as a consequence of an error-prone recombinational repair. The bacteria all have a histidine requirement that allows for the detection of revertants on minimal medium-plates. Stock cultures of each strain have been grown and checked to confirm their mutant characteristics. Stock cultures are prepared in nutrient broth with DMSO and frozen at -70°C.

Strain Designation	Gene Affected	Additional Mutations			Mutation Type Detected
		LPS	Repair	R Factor	
TA98	his D	rfa	uvr B	pKM101	Frameshift
TA100	his G	rfa	uvr B	pKM101	Base-pair substitution
TA1535	his G	rfa	uvr B	--	Base-pair substitution
TA1537	his C	rfa	uvr B	--	Frameshift
TA1538	his D	rfa	uvr B	--	Frameshift

B. Routine Check of Bacterial Strains for Mutations

Prior to initiation of the program all five of the *Salmonella* strains are tested for their specific types of mutation.

- Histidine Requirements. Cultures of all strains are streaked on minimal agar plates both with and without histidine. Biotin (0.1 ml of 0.5 mM/plate) is added since it is needed for growth by strains lacking the DNA repair system. All strains will grow only on the plates with histidine added.

- Deep Rough (rfa) Character. An agar overlay containing approximately 10^8 bacteria is added to nutrient agar plates. Filter paper discs containing 10 µl of 1 mg/ml crystal violet are placed on the top agar and, after 12-hr incubation, a clear zone of inhibition should be observed indicating the rfa mutation.

- Ampicillin-Resistant R Factor. A small amount of ampicillin solution (10 µl of 8 mg/ml in 0.02 N NaOH) is streaked across the surface of a nutrient agar plate. After drying, the TA98 and TA100 strains are cross-streaked against the ampicillin solution. The strains containing the R factor do not show growth inhibition around the ampicillin streak.

- Excision Repair System. The uvrB excision repair system, which is lacking in these strains, is confirmed by checking the UV sensitivity. Cultures are streaked on nutrient agar plates and half of each is irradiated (Sylvania G15T8 germicidal lamp) for 6 sec (TA1535, TA1537, TA1538) to 8 sec (TA98, TA100) at a distance of 33 cm. After incubation at 37°C for 24 hr, the strains without the repair system grow only on the unirradiated side of the plate.

- Spontaneous Reversion. Spontaneous reversion should be low. If a high reversion rate is observed a VBE agar plate is overlayed with top agar containing biotin-histidine to which has been added 0.05 ml of a 24-hr nutrient broth culture of the strain in question. Following incubation for 48 hr at 37°C approximately 10 colonies are isolated. These isolates are grown on nutrient agar plates and new tester strains isolated from the plates with low reversion rates.

C. Chemicals and Test Substances

Dilutions of all components, including the positive control mutagens (Tables 1 and 2) and solvent controls are prepared in unused pyrex glass tubes. We recommend that the five test substance dose levels shown in the dilution schedule be used. All substances are freshly prepared the day prior to use since compound stability often is unknown. The four control and the test compounds are stored according to suppliers directions regarding temperature, humidity and protection from light upon receipt. If specific directions are not received, the compounds are stored at 2°C in the dark. As a negative control, hemagglutination buffer (HA buffer) is used at a level of 100 ul/plate. Dilutions are prepared in distilled H_2O or DMSO* (Mallinckrodt, Spectr-AR) and in the plate assay used in the test within 1 hour. For the spot test, test-substance-impregnated discs are prepared the night prior to use and stored in hermetically sealed vessels. Glucose-6-phosphate and NADP used in preparation of the S-9 liver homogenate mixture are obtained from Sigma Chemical Co. or Boehringer Mannheim Co. The liver homogenate used in the preparation of the S-9 is obtained from Litton Bionetics, Inc.

D. Induction of Rat Liver Enzymes and Preparation of Liver Homogenates

For preparation of liver homogenates, Litton Bionetics uses male Sprague-Dawley rats (180-200 grams). To induce elevated levels of enzymes of the mixed function oxygenase system in the liver, the rats receive a single intraperitoneal injection of Aroclor 1254 (diluted in corn oil to 200 mg/ml) at a concentration of 500 mg/kg five days prior to sacrificing. Preparation of the S-9 fraction is with the procedure of Ames et al., Mutation Res. 31, 347-364, 1975, in which the homogenate is centrifuged at 9,000 xg and the supernatant which is the S-9 fraction, removed. Upon receipt at IITRI the S-9 fraction is stored at -70°C until used.

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* DMSO has been shown not to be toxic or mutagenic to the five test *Salmonella typhimurium* strains. Mutation Res. 31, 347-364, 1975, Ames, et al.

TABLE 1

REVERSION OF STANDARD TESTER STRAINS WITH MUTAGEN CONTROLS

Spontaneous Revertant Standard Count Without S-9

Culture Strain	No. Revertants
TA98	20-50
TA100	120-200
TA1535	10-35
TA1537	3-15
TA1538	15-35

Non-Activated System

Strain	Compound	Conc.	Approximate Number of Revertants	Range
TA98	2-Nitrofluorene (2NF) in DMSO	10 µg	700	600-800
TA100	Sodium Azide (NAZ) in H_2O	10 µg	950	850-1050
TA1535	Sodium Azide (NAZ) in H_2O	10 µg	975	875-1075
TA1537	9-Aminoacridine (9A) in Ethanol	100 µg	2150	1900-2400
TA1538	2-Nitrofluorene (2NF) in DMSO	10 µg	650	550-750

Activated System

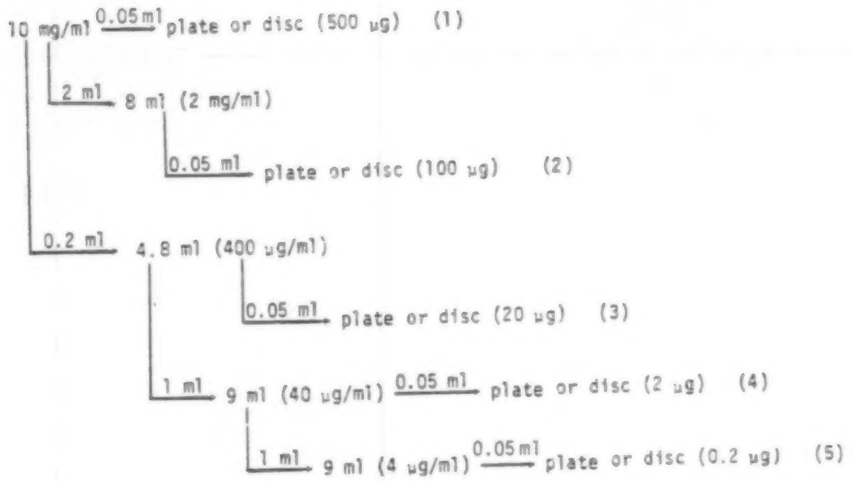
Strain	Compound	Conc.	Approximate Number of Revertants	Range
TA98	2-Anthramine (2A) in DMSO	10 µg	1650	1500-1800
TA100	2-Anthramine (2A) in DMSO	10 µg	1900	1700-2100
TA1535	2-Anthramine (2A) in DMSO	10 µg	300	250-350
TA1537	2-Anthramine (2A) in DMSO	10 µg	150	125-175
TA1538	2-Anthramine (2A) in DMSO	10 µg	1900	1700-2100

TABLE 2

GENERAL TEST CHEMICAL DILUTION SCHEMES

TEST CHEMICAL

Normally Used Scheme



10 mg/ml $\xrightarrow{0.05\ ml}$ plate or disc (500 µg) (1)

$\xrightarrow{2\ ml}$ 8 ml (2 mg/ml)

$\xrightarrow{0.05\ ml}$ plate or disc (100 µg) (2)

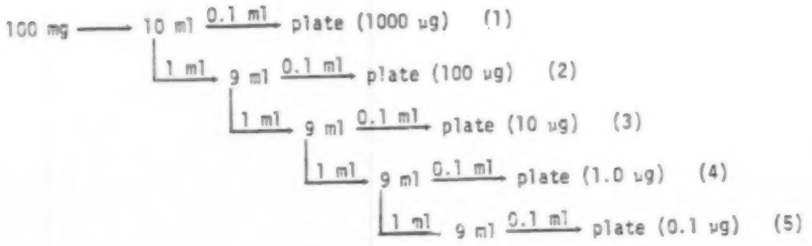
$\xrightarrow{0.2\ ml}$ 4.8 ml (400 µg/ml)

$\xrightarrow{0.05\ ml}$ plate or disc (20 µg) (3)

$\xrightarrow{1\ ml}$ 9 ml (40 µg/ml) $\xrightarrow{0.05\ ml}$ plate or disc (2 µg) (4)

$\xrightarrow{1\ ml}$ 9 ml (4 µg/ml) $\xrightarrow{0.05\ ml}$ plate or disc (0.2 µg) (5)

Alternate Scheme



100 mg ⟶ 10 ml $\xrightarrow{0.1\ ml}$ plate (1000 µg) (1)

$\xrightarrow{1\ ml}$ 9 ml $\xrightarrow{0.1\ ml}$ plate (100 µg) (2)

$\xrightarrow{1\ ml}$ 9 ml $\xrightarrow{0.1\ ml}$ plate (10 µg) (3)

$\xrightarrow{1\ ml}$ 9 ml $\xrightarrow{0.1\ ml}$ plate (1.0 µg) (4)

$\xrightarrow{1\ ml}$ 9 ml $\xrightarrow{0.1\ ml}$ plate (0.1 µg) (5)

TABLE 2 (continued)

EFFLUENT OR WASTEWATER (Filter sterilized)

Direct $\xrightarrow{0.05\ \text{ml}}$ conc./disc or plate (1)

$\xrightarrow{1\ \text{ml}}$ 9 ml $\xrightarrow{0.05\ \text{ml}}$ 1:10/disc or plate (2)

$\xrightarrow{1\ \text{ml}}$ 9 ml $\xrightarrow{0.05\ \text{ml}}$ 1:100/disc or plate (3)

$\xrightarrow{1\ \text{ml}}$ 9 ml $\xrightarrow{0.05\ \text{ml}}$ 1:1000/disc or plate (4)

$\xrightarrow{1\ \text{ml}}$ 9 ml $\xrightarrow{0.05\ \text{ml}}$ 1:10,000/disc or plate (5)

E. Preparation of Liver Homogenate (S-9) Reaction Mixture

The S-9 mix is prepared by adding the following compounds on a per ml basis: 0.1 ml, S-9 fraction; 0.45 ml salt solution (8 μmoles $MgCl_2$, 33 μmoles KCl) and phosphate buffer (100 μmoles), pH 7.4; and 0.45 ml NADP-glucose-6-phosphate solution (5 μmoles of glucose-6-phosphate and 4 μmoles NADP). This mixture is made fresh each day the mutagenicity test is run. The S-9 mix solution is sterilized through a 0.45μ filter (Falcon No. 7102) and the S-9 fraction added just prior to testing.

F. Preparation of Medium and Agar Overlay

The minimal glucose agar medium plates used throughout the study contain 1.5% Difco-Bacto agar in Vogel-Bonner medium E (Vogel and Bonner, J. Biol. Chem. 218, 97-106, 1956) with 2% dextrose (Table 3). Each petri dish contains approximately 20 ml of minimal agar medium. Top agar, 0.6% Difco agar and 0.5% NaCl, is autoclaved. When ready for use, 10 ml of a filter sterilized solution of 0.5 mM-L-histidine-HCL and 0.5 mM biotin is added/100 ml. The materials are mixed by gentle swirling of the flask and 2 ml added to 16 x 150 mm disposable glass test tubes and allowed to equilibrate to 45°C in a water bath before addition of any bacteria or chemicals.

G. Mutagenesis Assay

The Ames protocol (Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian Microsome Mutagenicity Test. Mutation Res. 31, 347-364, 1975 and Ames Laboratory update 3/79) is used.

1. Plate Incorporation Test

Prior to the assay and optional at additional cost, the chemical under test is examined at each dilution for toxicity against each of the five tester strains, both activated and non-activated. After 24 hours incubation with shaking at 37°C, 0.1 ml of each tester strain is spread, using sterile glass streakers, on duplicate Oxoid nutrient agar plates that have been dried overnight. In the activated system, duplicate nutrient agar plates are overlayed with approximately 2.6 ml of top agar overlay that contains 0.1 ml of culture and the S-9 mix only in the same

TABLE 3

MUTAGENICITY TEST MEDIA

VOGEL-BONNER E MINIMAL MEDIUM (VBE AGAR)

Bacto-Agar Agar	15g	(18g)
Dextrose	20g	(24g)
H_2O	980 ml	(1176 ml)

Autoclave, cool, add 20 ml (24 ml) of sterile Vogel-Bonner E medium 50X. Pour 20 ml/plate.

VOGEL-BONNER E MEDIUM 50X

$MgSO_4 \cdot 7H_2O$	10g
Citric Acid·H_2O	100g
K_2HPO_4	500g
$NaNH_4HPO_4 \cdot 4H_2O$	175g
H_2O	670 ml

Dissolve each completely in the order listed, autoclave for 12-15 min. Final volume is 1L. Store in refrigerator.

NUTRIENT BROTH

Oxoid-Nutrient broth #2	25g
H_2O	1L

For organism preparation, 20 ml is aseptically added to sterile 50 ml flasks.

Distribute in 100 ml quantities and autoclave.

TOP AGAR OVERLAY

Bacto-Agar Agar	6g
NaCl	5g
H_2O	1L

D-GLUCOSE-6-PHOSPHATE + NADP

NADP (4mM) mw 765.4	3.06 mg/ml
D-Glu.(5mM) mw 282.1	1.41 mg/ml

total volume 1 ml

$MgCl_2$-KCl SOLUTION

$MgCl_2 \cdot 6H_2O$	6.5g	(32mM)
KCl	9.84g	(132mM)
H_2O	1L	

Autoclave in 500 ml quantities. store in refrigerator.

0.4M PHOSPHATE BUFFER pH 7.4

$NaH_2PO_4 \cdot H_2O$	55.2g	(0.4M) $\xrightarrow{qs}$ 1L
$Na_2HPO_4 \cdot 7H_2O$	107.2g	(0.4M) $\xrightarrow{qs}$ 1L

Add 95 ml of NaH_2PO_4 solution + 405 ml Na_2HPO_4 solution for a total of 500 ml, autoclave, store in refrigerator.

TABLE 3 (cont.)

PHOSPHATE BUFFER - $MgCl_2$-KCl MIX SOLUTION

Cool $MgCl_2$-KCl solution sterile
Cool 0.4 m Phosphate buffer sterile
> mix equal quantities of each

BIOTIN-HISTIDINE SOLUTION

D-Biotin	12.22 mg	(0.5 mM)	mw 244.3
L-Histidine HCL	10.58 mg	(0.5 mM)	mw 211.6
H_2O	100 ml		

Filter sterilized in ice bath and stored @ -20°C

S-9 MIX SOLUTION

0.4M PO_4 Buffer	20 ml
$MgCl_2$-KCl solution	20 ml
NADP	244.8 mg
D-Glu.-6-PO_4	112.8 mg
H_2O	40 ml

Filter sterilized in ice bath and stored @ -20°C concentration used in the mutagenesis assay. A sterile filter paper disc (S&S 740-E) impregnated with 0.05 ml of the test chemical is placed onto the surface of the agar or overlay agar and allowed to stand for about 15 minutes before the plates are incubated inverted at 37°C. The plates are read after 24 hours incubation and if negative are reincubated for an additional 24 hours. The diameter of any zones of inhibition is then measured with the unaided eye to the nearest millimeter (mm) from the bottom of the plate. Toxicity at the highest dose level necessitates lowering of the maximum dose level so that there is only slight inhibition to the tester strain; additionally a low dose level is chosen that is well below the concentration that has been demonstrated to be toxic.

The quantitative plate incorporation assay is outlined in Figure 1. As a guide to observing good laboratory practices, the DHEW/FDA "Regulations for Good Laboratory Practices" (Fed. Reg. Vol. 43, No. 247, Friday, December 22, 1978) is followed for all testing. Pour plates are made by adding, in order, 0.1 ml of a 24-hr nutrient broth culture (incubated with shaking in 50 ml flask) of the tester strain to 2 ml of molten top-agar (45°C) in sterile 16 x 150 mm test tubes followed by 0.05 or 0.1 ml of the control or unknown chemical to be tested and 0.5 ml of the described S-9 mix kept in ice bath. The small amount of histidine and biotin added to the top agar allows the bacteria to undergo several divisions, in many cases a necessity for mutagenesis. The tubes are mixed by rotating between the palms of the hands and poured onto the surface of a minimal agar plate. All plates are dried overnight prior to use. The plates are tilted quickly so the entire surface of the agar plate is covered with top agar. This procedure is minimized to no more than 20 seconds since the enzyme activity in the S-9 mix can be inactivated rapidly. In addition to the test plates, control plates for spontaneous reversion rate are made for each tester bacterial strain in which the substance being tested is omitted. All petri dishes are incubated inverted at 37°C for 2 days, after which the number of revertant colonies per plate is counted. As a check for contamination, sterility tests are made on all mutagen and test

SCHEME

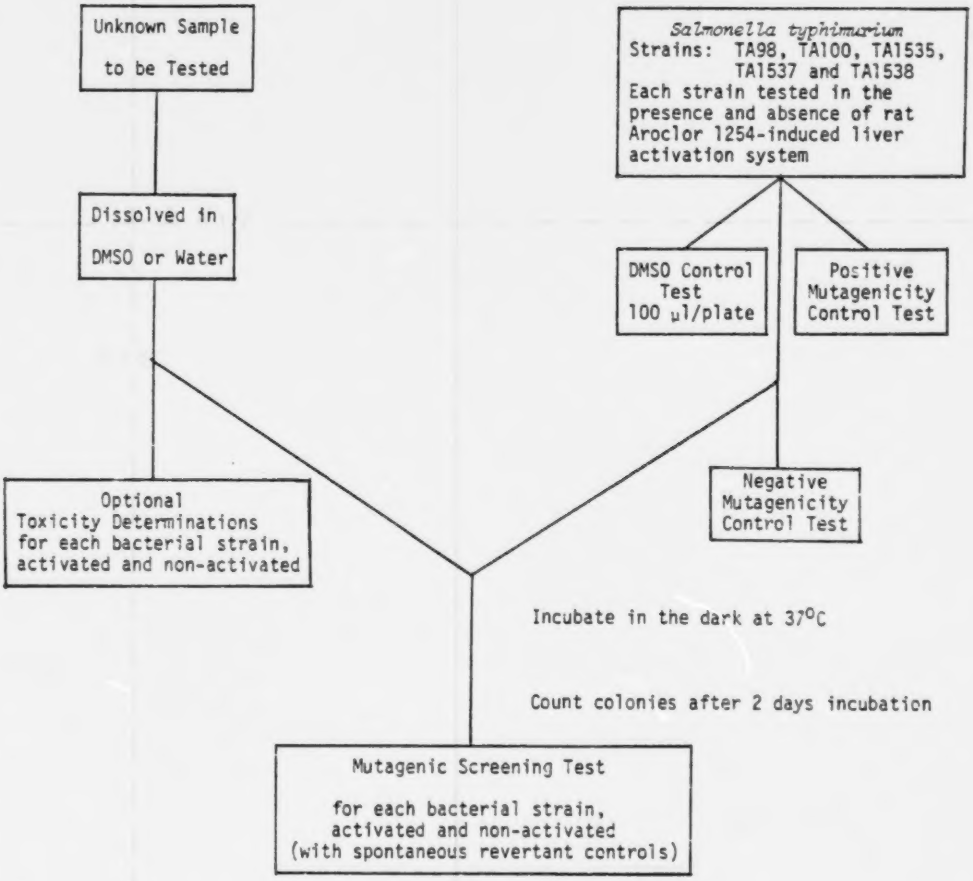

Unknown Sample to be Tested
Dissolved in DMSO or Water
Optional Toxicity Determinations for each bacterial strain, activated and non-activated
Salmonella typhimurium
Strains: TA98, TA100, TA1535, TA1537 and TA1538
Each strain tested in the presence and absence of rat Aroclor 1254-induced liver activation system
DMSO Control Test 100 µl/plate
Positive Mutagenicity Control Test
Negative Mutagenicity Control Test
Incubate in the dark at 37°C
Count colonies after 2 days incubation
Mutagenic Screening Test
for each bacterial strain, activated and non-activated (with spontaneous revertant controls)

Figure 1. Procedure for Salmonella Mutagenicity Screening Test (Plate Assay)

SCHEME

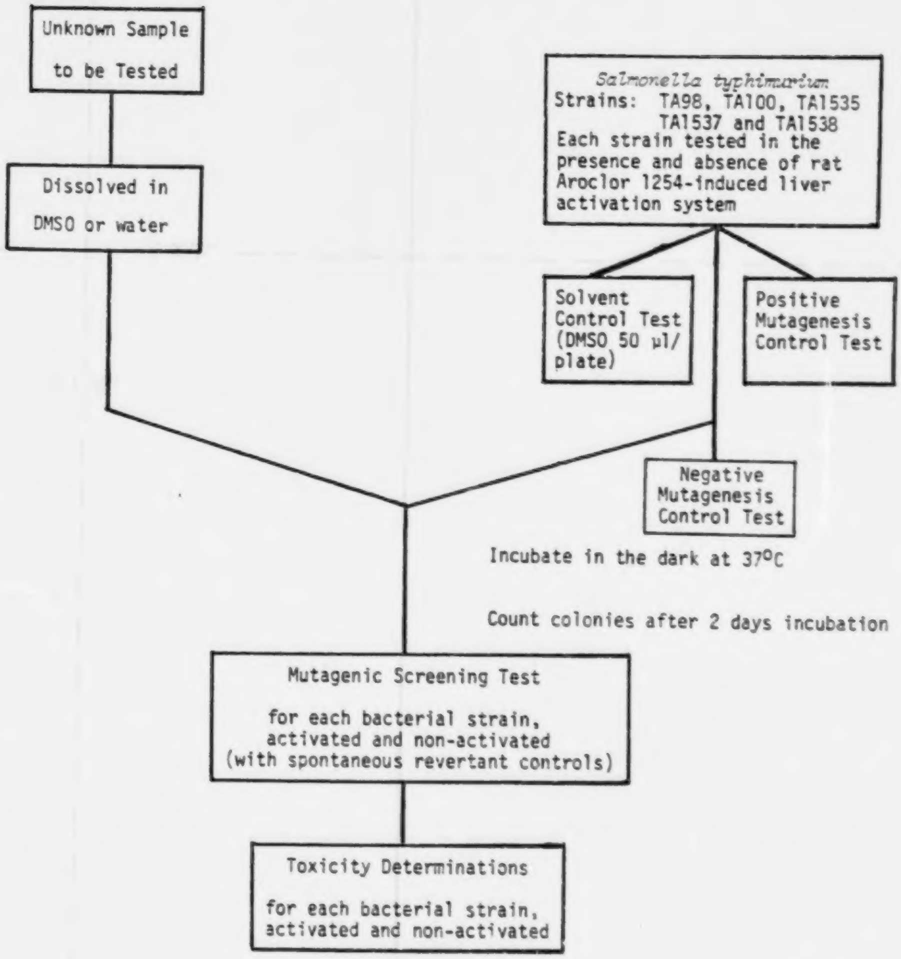

Unknown Sample to be Tested
Dissolved in DMSO or water
Salmonella typhimurium
Strains: TA98, TA100, TA1535 TA1537 and TA1538
Each strain tested in the presence and absence of rat Aroclor 1254-induced liver activation system
Solvent Control Test (DMSO 50 µl/plate)
Positive Mutagenesis Control Test
Negative Mutagenesis Control Test
Incubate in the dark at 37°C
Count colonies after 2 days incubation
Mutagenic Screening Test
for each bacterial strain, activated and non-activated (with spontaneous revertant controls)
Toxicity Determinations
for each bacterial strain, activated and non-activated

Figure 2. Procedure for Salmonella Mutagenicity Screening Test (Spot test)

substance solutions by incorporating a 0.1 ml of solution into a top agar overlayed minimal agar plate without bacteria or S-9 and incubating at 37°C for 48 hours. In addition, 5% of the Vogel-Bonner E medium agar plates are overlayed with overlay agar, biotin-histidine and S-9 at the same time the test is run and incubated at 37°C for 48 hours to check for contamination. All test and control plates are in triplicate.

2. Spot Test

The protocol to be used for the spot test mutagen assay is outlined in Figure 2. Pour plates are made by adding, in order, 0.1 ml of a 24 hour nutrient broth culture (incubated w/shaking in 50 ml flask) of the tester strain to 2 ml of molten top agar (45°C) in sterile 16 x 150 mm test tubes, followed by 0.5 ml of the described S-9 mix kept in ice bath. In the case of the control mutagens, 0.1 ml of each is added to the tubes of overlay agar following addition of the tester strain. The small amount of histidine and biotin added to the top agar will allow the bacteria to undergo several divisions, a necessity in many cases for mutagenesis. The tubes are mixed by rotating between the palms of the hands and poured onto the surface of a minimal agar plate. All plates are dried overnight prior to use. The plates are tilted quickly so the entire surface of the agar plate is covered with top agar. This procedure is minimized to no more than 20 sec. since the enzyme activity in the S-9 mix can be inactivated rapidly. After the agar has hardened, approximately 15 min, sterile filter paper discs (S&S 740-E) containing the chemicals under test are placed on the agar surface. (If necessary, 5 mg of test chemical can be spotted directly on the agar). In addition to the test plates, tester strain control plates for spontaneous reversion rate are made for each tester bacterial strain in which the substance being tested is omitted. All petri dishes are incubated inverted at 37°C for 2 days, after which the number of revertant colonies are counted. As a check for contamination, sterility tests are made on all mutagen and test substance solutions by incorporating a 0.1 ml of solution into a top agar overlayed minimal agar plate without bacteria or S-9 and incubating at 37°C for 48 hours. In addition, 5% of the Vogel-Bonner E medium agar plates are overlayed with overlay agar, biotin-histidine and S-9 at the same time the test is run and incubated at 37°C for 48 hours to check for contamination. All test and control plates are in triplicate. Test chemical toxicity is determined by inhibition of the bacterial background lawn evidenced by zones of inhibition, measured from the bottom of the plate to the nearest mm with the unaided eye.

H. Test Results and Analysis

A test is considered positive if the number of mutant colonies on the test chemical treated dishes are significantly greater than the number on control (spontaneous revertants) dishes. From mutagenicity data tabulated by Dr. Ames (Mut. Res. 31, 347-364, 1975), a simple rule of thumb may be applied. If a chemical is to be considered positive, it should have double the number of mutants per plate, over the spontaneous revertants. Statistical treatment of the number of mutant colonies on chemical-treated plates compared to spontaneous revertants have repeatedly shown in the Ames test that a 2-fold increase in bacterial counts is a positive test. A negative result may not necessarily mean the compound is non-mutagenic but that the mutagenic effect may be below the level detectable in this system.

I. Data Handling

All original data are recorded in ink in prenumbered bound logbooks. All data entries are dated and signed or initialed by the person entering the data on the day of entry. Any change in data entries is accompanied by the date and signature or initial of the person making the change and the reason for such change. Upon completion of the study all raw data are submitted to the Life Sciences Archives for storage.

III. TIME ESTIMATE

It is estimated that completion of the study including report submission will require a period of approximately 6 weeks from initiation of the program. If toxicity determinations in the plate incorporation assay are desired, an additional week will be necessary.

IV. REPORTS

Within 3 weeks of completion of the study a written report, including tabulation of individual colony counts, the average of three plates, and summary and analysis of results, is submitted to the sponsor. Included is a statement of the Quality Assurance Unit. If requested by the sponsor, results of the study can be telephoned directly after completion of each test substance or group of substances. This however will not replace a written report. Further, the sponsor's representatives are welcome to visit IITRI and inspect facilities, procedures, or records relative to the sponsor's study during the course of the study.

V. QUALITY ASSURANCE PROGRAM

The Life Sciences Division has developed an internal audit system to assure compliance with the FDA Good Laboratory Practices Regulations. A Quality Assurance Unit under the direct supervision of the Division Director monitors laboratory performance and results to assure that protocols and written standard operating procedures are being followed.

The Quality Assurance Unit conducts inspections at least every three months on any study lasting more than six months. For shorter studies audit schedules are planned prior to initiation of the work. The Section Head responsible for the specific project assures that the proper information (facilities, equipment, personnel, methods, procedures, protocols and records) is supplied to the Unit. Each critical phase of a study or procedure is reviewed and the inspection is documented by written reports submitted to management. These reports give the status of each study, and the problems and the actions taken to resolve them. These in-depth evaluations detect, at an early stage, errors in recording data, unusual patterns of events, lack of conformity to protocol and deviations from standard operating procedures. Changes from approved protocol requires explanation and proper authorization. In addition, a Master Schedule sheet of ongoing studies is kept by the Supervisor of Quality Assurance showing the required GLP information.

VI. PROJECT MANAGEMENT

Life Sciences Division projects are managed by assigned Project Leaders who report directly to a Section Head. The Section Head provides the overall administrative and scientific direction. The Project Leader is responsible for all aspects of a program. This person develops the project plan or protocol as well as standard operating procedures and directs all project-related activities, schedules testing, studies the data and analyses, and prepares the reports. Contact with the sponsoring organization is maintained through the Project Leader. Where capabilities outside the section or Life Sciences Division are required, the staff of other IITRI Divisions and outside organizations may be consulted.

To assess progress and to plan project activities, the Project Leader and Section Head meet informally at frequent intervals. The review includes project staff members and the Director of Life Sciences as necessary. All reports are reviewed by the Section Head before submission.

APPENDIX M

Laboratory: Jefferson Professional Services
P.O. Box 3397
Little Rock, AR 72203

Submitter: Ms. Shirley Louie
Microbial Geneticist
(501)374-1296

Date of submission: December 8, 1980

Omissions: None

Jefferson Professional Services
Scientific Consulting · Microbiological Analysis · Chemical Analysis
Microbiological Mutagenesis Testing · Managerial Administrative Support

December 8, 1980

Ms. Stephanie Toney
Genetic Bioassay Branch (MD 68)
United States Environmental
Protection Agency
Health Effects Research Laboratory
Research Triangle Park, N.C. 27711

Dear Ms. Toney:

Enclosed is the protocol for the Ames Salmonella/Microsome Assay for Bacterial Mutagenesis used at Jefferson Professional Services. I would appreciate a final copy of the compilation of protocols when it becomes available.

If I can be of any further assistance, please feel free to notify my office.

Sincerely,

Ms. Shirley Louie
Microbial Geneticist

SL/rj

ENC.

P.O. Box 3397 Little Rock, Arkansas 72203 (501) 374-1296

AMES SALMONELLA/MICROSOME ASSAY FOR BACTERIAL MUTAGENICITY: QUANTITATIVE POUR PLATE PROCEDURE

Purpose: The Ames Mutagenicity Assay is designed to provide an evaluation of the mutagenic potential of chemicals using mutant strains of Salmonella typhimurium as indicator organisms.

Test System Justification: The Ames Mutagenicity Assay, an in vitro short term test used to detect point mutations, has been selected as one of a battery of assays used in conducting mutagenicity determinations. (1)

Test Organisms: Salmonella typhimurium strains TA-1535, TA-1537, TA-1538, TA-98 and TA-100 are used. All strains are received from Dr. Bruce Ames, University of California at Berkeley and stored at -70°C.

The strains used in this study have a deletion through the uvrB region of the chromosome resulting in a lack of excision repair and an rfa mutation which has resulted in the removal of the lipopolysaccharide coat distal to the ketodeoxyoctanoate-lipid core, making the cells more permeable to compounds with large molecular configurations. The lack of excision repair capability makes the organisms more sensitive to DNA damage which otherwise might be corrected by the excision repair process.

Strains TA-1535 and TA-100 are missense mutants which are reverted by mutagens which cause base-pair substitutions. Strains TA-1537, TA-1538 and TA-98 are frameshift mutants.

TA-100 and TA-98 are sub-strains of TA-1535 and TA-1538, respectively. These sub-strains have the addition of an R-factor plasmid, pkm 101, which confers resistance to ampicillin and increases their sensitivities to mutagens. It also enables TA-100 to detect both frameshift and base-pair substitution mutagens. (2)

Test Article Preparation: Solutions of test article(s) are prepared in deionized water, 0.8% sodium chloride or 100 mM phosphate buffer, ph 7.4. If test material(s) do not dissolve in any of the above mentioned solvents, freshly prepared solutions in dimethylsulfoxide are used.

Test Methodology:

A. Materials

1. Media

Vogel-Bonner-Glucose Mutation Agar Medium (3)

$MgSO_4 \cdot 7H_2O$	0.2 gm
Citric Acid $\cdot$ H_2O	2.0 gm
K_2HPO_4 anhydrous	10.0 gm
$NaNH_4PO_4 \cdot 7H_2O$	3.5 gm
Agar	15.0 gm
H_2O	1000.0 ml

The medium is autoclaved and supplemented with two percent (2%) sterile glucose prior to pouring plates.

Top Agar Medium

Agar	0.6 gm
NaCl	0.6 gm
H_2O	100.0 ml

Top agar is autoclaved and supplemented with 10.0 mls of a solution containing 0.5 mM L-histidine and 0.5 mM biotin.

Nutrient Broth

Sterile nutrient broth is used for overnight growth of indicator organisms.

0.15 M KCl Solution

KCl	11.2 gm
distilled H_2O	1000.0 mls

This solution is sterilzed by autoclaving.

Histidine-Free Agar Medium

Vogel-Bonner-glucose mutation agar containing 0.05 mM biotin is used to verify the genotype of the histidine revertants.

Nutrient Agar Medium

Nutrient agar is used to verify viability of the revertant colonies which are plated on hisitidine-free medium.

2. Microsomal Supernatant Fraction (S-9)

Induction of Rat Liver Enzymes

Male Sprague Dawley rats (300 grams each) are used for the induction of liver enzymes. A single intraperitoneal injection of aroclor 1254 (diluted in corn oil to a concentration of 200 mg/ml) at a dose of 500 mg/kg body weight is given to each rat five (5) days before sacrifice. The rats are given drinking water and feed *ad libitum* until 24 hours before sacrifice, at which time, the food is removed. On the fifth day of induction, rats are killed by gassing with CO_2.

Preparation of Liver Homogenate (Lot# AS-1)

All steps are performed at 0-5°C using ice baths, sterile solutions and glassware. The livers are removed with sterile scissors and forceps and placed in preweighed sterile petri plates. Liver weights are determined and the livers washed with sterile 0.15M KCl solution. Livers are then transferred to a beaker containing 3.0 mls of 0.15M KCl solution per gram wet liver. Livers are minced with sterile scissors and homogenized in a Potter-Elvehjan apparatus with a Teflon pestle. The homogenate is centrifuged for 15 minutes at 9,000 x g and the supernatant is decanted and saved. Protein content and sterility are determined. The S-9 fraction is quick frozen and stored at -70°C.

Preparation of S-9 Mixture

For each milliliter of S-9 mixture, 0.3 ml of S-9 fraction is combined with 0.7 ml of a solution containing: 8 mM $MgCl$, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP (nicotinamide adenine dinucleotide phosphate) and 100 mM sodium phosphate buffer, pH 7.4. The S-9 mixture is kept in an ice bath during the test.

The activity of the S-9 mixture is confirmed by its ability to activate 2-acetylaminofluorene, a known mutagen which requires mammalian enzyme activation to form reactive metabolites.

3. Controls

Negative Controls

0.1 ml of each bacterial strain (1-2 x 10^8 cells) serves as the untreated negative control. 0.1 ml of bacteria plus 0.1 ml of dimethylsulfoxide serves as the treated negative control as well as the solvent control. Each of these controls is tested for all five strains of bacteria, both with and without mammalian metabolic activating enzymes (S-9).

Positive Controls Used in the Ames Assay Without Activation

Indicator Strain	Positive Control Mutagen	Concentration (μg/plate)	Probable Mutagenic Activity
TA-1535	sodium azide	5.0	Base-pair substitution
TA-1537	9-aminoacridine	50.0	Frameshift mutation
TA-1538	2-nitrofluorene	1.0	Frameshift mutation
TA-98	2-nitrofluorene	1.0	Frameshift mutation
TA-100	sodium azide	5.0	Base-pair substitution

Positive Controls Used in the Ames Assay With Activation

Indicator Strain	Positive Control Mutagen	Concentration (μg/plate)	Probable Mutagenic Activity
TA-1535	2-anthramine	1.0	Base-pair substitution
TA-1537	2-anthramine	1.0	Frameshift mutation
TA-1538	2-anthramine	1.0	Frameshift mutation
TA-98	2-anthramine	1.0	Frameshift mutation
TA-100	2-anthramine	1.0	Frameshift mutation

All positive control chemicals are dissolved in dimethylsulfoxide (DMSO) and amounts are added in 0.1 ml volumes.

B. Procedures

Toxicity Determination

The effect of the test article on the survival of a representative bacterial strain is determined prior to the mutagenicity assay. Strain TA-100 is chosen since its background revertant number is high and decreases in total cell numbers can be detected by quantitative decreases in the number of background revertants as well as by a thinning or disappearance of the background lawn. Equal volumes of varying concentrations of compund #203-1A are added to serile, capped 12x75 mm test tubes each containing 2.0 mls of molten top agar heated at 46°C along with either 0.5 ml of 0.15 M KCl or 0.5 ml of S-9 mixture and 0.1 ml (1-2x10^8 of cells) an overnight culture of strain TA-100. The contents of each tube is overlayed onto Vogel-Bonner base agar plates, allowed to solidify, inverted and incubated at 37°C for 72 hours. Resulting colonies are counted. All platings are done in replicate of at least two.

Mutagenicity Assay - Pour Plate Procedure

1. Dilute test article and prepare test doses.
2. Prepare fresh top agar.
3. Place 2.0 mls of sterile molten top agar supplemented with histidine-biotin into sterile 12x75 mm capped test tubes preheated to 46°C in a dry heating block.
4. Allow agar temperature to equilibrate to 46°C.
5. Thaw S-9 fraction at room temperature.
6. Combine 3 parts S-9 fraction with 7 parts NADPH regenerating solution to make S-9 mixture. (Keep in ice bath during test).
7. To each tube of agar, add the following:
    a. No addition, 0.1 ml DMSO, 0.1 ml of test article solution of a given concentration or 0.1 ml of control chemical solution.
    b. 0.5 ml of 0.15 M KCl solution or 0.5 ml of S-9 mixture.
    c. 0.1 ml of tester strain.
8. Overlay top agar onto Vogel-Bonner base plates.

9. Allow agar to solidify, invert plates and incubate at 37°C for 72 hours.

10. Count resulting colonies.

All platings were done in replicates of at least two.

Verification of Revertant Colonies

When positive mutagenic results are obtained, the genotype of revertant colonies are checked by picking representative colonies at random and stabbing them onto histidine-free medium and then onto nutrient agar. Plates are inverted and incubated at 37°C for 24 hours. Plates are examined for bacterial growth in the stab marks.

C. Results

The results include the means and standard deviations for the toxicity determination data and the mutagenicity data obtained for each dose level of the negative controls, positive controls and test article assayed. Also included are the statistics from the linear regression analysis to determine the strength of the dose response obtained with each tester strain.

D. Discussion

In evaluating the validity of the controls, the following criteria must be met:

1. All control replicate plates must have approximately equal numbers of revertant colonies.
2. Solvent controls must have approximately the same number of colonies as the spontaneous reversion controls.
3. Positive controls must give at least three times the number of colonies as the spontaneous reversion controls.

The following criteria are used to evaluate the results with an unknown test article:

1. To be considered a positive mutagen, a test article should exhibit a dose response effect. There should be an increasing number of mutants observed with increased amount of test article.

2. To score a positive response for strains TA-1535, TA-1537 and TA-1538, a dose response effect must be evident over at least three concentrations separated by at least one half log steps. The minimum dose response should be at least two times the solvent control response.
3. To score a positive response for strains TA-98 and TA-100, a dose response must be observed over three concentrations separated by at least one half log steps. The maximum response should be at least two times the solvent control values (4).

Qualitative evaluations of toxicity can be made by examining the background lawn of each plate. The appearance of tiny pinpoint colonies in the background lawn between the revertant colonies indicates that the test article is toxic to the indicator organisms at that particular dose. The highest dose chosen for the Ames Salmonella/Microsome Assay should show toxicity. The lower doses should show little or no toxicity. Evaluations of mutagenic activity cannot be made at toxic dose levels since cell death could result in a decrease in the number of revertant colonies or cause the appearance of nonrevertant colonies which form because of histidine available from cell debris.

E. Statistical Analysis of Test Data

A least squares linear regression analysis is used to compute the "best fit" regression line for response vs. dose level for the test article (5). Regression analyses are not performed on data from tester strains when less than three nontoxic dose levels were available. The individual regression lines represent the strength of the dose response relationship obtained with each bacterial tester strain. For each response line,

$$y = mx + b$$

a correlation coefficient, r, a t value of the slope and probability function, P (t), are computed to test the hypothesis

H_0: the data do not suggest mutagenic activity against

H_1: the data indicate a positive mutagenic response

At the 95% confidence limit, $0.025 \leq P(t) \leq 0.975$ supports the null hypothesis.

General Considerations: Each study will be conducted in accordance with good laboratory practices, including record keeping and appropriate internal quality assurance controls.

If the need should arise for changes in the approved protocol after the initiation of the study, these changes will immediately be reported to the sponsor. The changes and reasons for the changes will be documented and will become a permanent part of the study file.

All information concerning each study will be kept confidential unless release of the information is approved in writing by the sponsor.

References:

1. Ames, B.N., Durston, W.E., Yamasaki, E. and Lee, F.D.: Proc. Natl. Acad. Sci. USA, 1973, 70(8):2281-2285.

2. Ames, B.N., Lee, F.D. and Durston, W.E.: Proc. Natl. Acad. Sci. USA, 1973, 70(3):782-786.

3. Vogel, H.J. and Bonner, D.M.: J. Biol. Chem. 1956, 218:97-106.

4. Strategies for Short-Term Testing for Mutagens/Carcinogens Ed. B.E. Butterworth, CRC Press, Inc., West Palm Beach, FA, 1979.

5. Snedecor, G.W. and Cochran, W.G.: Statistical Methods, Sixth Ed., Iowa State University Press, 1967, p. 153.

APPENDIX N

Laboratory: Litron Laboratories, Ltd.
1351 Mount Hope Ave.
Rochester, NY 14620

Submitter: Dr. Andrew M. Tometsko
Director of Research
(716)275-4008

Date of submission: January 22, 1981

Omissions: Price List for Ames Test

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Litron
Laboratories
Ltd.

1351 Mount Hope Avenue Rochester New York 14620
phone 716 275 4008

January 22, 1981

Ms. Stephanie Toney
Genetic Bioassay Branch (MD 68)
United States Environmental Protection Agency
Health Effects Research Laboratory
Research Triangle Park
North Carolina 27711

Dear Ms. Toney:

Thank you for your letter of January 16, 1981 concerning your compilation of Ames Test Protocols. Our protocol is essentially that of Ames et al (Ames, B., J. McCann and E. Yamasaki, Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-microsome mutagenicity test, Mut. Res. 31 (1975) p. 347-364). Enclosed is a description of the protocol that we use. I hope you find this information useful in your work. I would appreciate receiving a copy of your final report when it is available.

Sincerely,

Andrew M. Tometsko
Andrew M. Tometsko, Ph.D.
Director of Research

AMT:crt
Encl.

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Litron
Laboratories
Ltd.

1351 Mount Hope Avenue Rochester New York 14620
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Ames Testing Protocols

GENETIC TOXICOLOGICAL INVESTIGATIONS:

The Salmonella typhimurium mutagenesis test

INTRODUCTION

There is evidence indicating that a significant fraction of human cancers arise from exposure to chemicals in the environment. Consequently, interest has centered on the development of *in vitro* tests for rapid identification of problem chemicals. Of these, the Ames *Salmonella typhimurium* reversion test is perhaps the best characterized to date (1,2,3). In this test, genetic activity of test chemicals is indicated by reversion of auxotrophic histidine mutants. Although the relationship between mutagenesis and neoplastic transformation is not well defined, there appears to be more than a circumstantial similarity. Ames has demonstrated a greater than 90% correlation between chemicals which are mutagenic in this test and those which induce tumors *in vivo* (2). Other investigators have indicated that this test may even give some indication of the magnitude of carcinogenic potential (4). It appears, then, that the *S. typhimurium* reversion test can be both sensitive and economical as an early indicator of problem chemicals.

TEST PROTOCOLS

1) BACTERIAL STRAINS

The *S. typhimurium* strains used in this study were obtained from Dr. Bruce Ames, University of California, Berkeley, California. The strains used and their genetic characteristics are given in Table 1.

TABLE 1

GENETIC CHARACTERISTICS OF INDICATOR STRAINS

		Additional Mutations				Type of Mutational Event
S. typhimurium	Gene Affected	DNA Repair	LPS	Biotin Requirement	R. Factor Plasmid	
TA98	*hisD*	*uvrB*	*rfa*	*bio-*	pKM101	frameshift
TA100	*hisG*	*uvrB*	*rfa*	*bio-*	pKM101	base-pair substitution
TA1535	*hisG*	*uvrB*	*rfa*	*bio-*	-	base-pair substitution
TA1537	*hisC*	*uvrB*	*rfa*	*bio-*	-	frameshift
TA1538	*hisD*	*uvrB*	*rfa*	*bio-*	-	frameshift

Each of these mutants is a histidine auxotroph. Revertants are easily identified as colonies which grow in the presence of low levels of L-histidine (0.05 *mM*). Both frameshift and base-pair substitution mutagenic events are represented in order to identify mutagens of both types. The additional genetic markers serve to made the test more sensitive for certain types of mutagens. The DNA repair mutation (*uvrB*) eliminates excision repair, an error free repair pathway for DNA damage induced by UV light and certain chemical mutagens. The presence of the *uvr* mutation makes this test more responsive for chemicals which induce damage which is normally corrected by excision repair. The *uvrB* mutation is a part of deletion mutation extending into a gene for biotin biosynthesis. Therefore, the biotin requirement is indicative of the deletion of this region. The *rfa* mutation changes the properties of the bacterial cell wall and facilitates the uptake of certain types of chemicals into these cells. The *rfa* mutation is indicated by sensitivity of the bacterial strains to deoxycholate. The R factor plasmid (pKM101) carried by TA98 and TA100 makes those strains more responsive to a variety of mutagens. The plasmid also carries an ampicillin resistance gene, so ampicillin resistance in TA98 and TA100 indicates that those strains retain the plasmid. Results of control experiments demonstrating the presence of these genetic markers are given in Table 2.

TABLE 2

INDICATION OF APPROPRIATE GENETIC MARKERS

STRAIN	-HISTIDINE	-BIOTIN	+DEOXYCHOLATE	+AMPICILLIN
TA98	no growth	no growth	sensitive	resistant
TA100	no growth	no growth	sensitive	resistant
TA1535	no growth	no growth	sensitive	sensitive
TA1537	no growth	no growth	sensitive	sensitive
TA1538	no growth	no growth	sensitive	sensitive

2) PLATING ASSAY

The cells are grown for 16 hours in nutrien broth (7.5gm Oxoid and 3mg histidine in 300ml H_2O) to stationary phase. For the plating assay, 0.1ml cells (10^8), mutagen (0.1ml solvent: DMSO, water, etc.), top agar (0.6% agar, 0.5% NaCl) supplemented with .05mM biotin, .05mM histidine, and, when necessary, 0.5ml S-9 are mixed and added to the surface of Vogel-Bonner medium plates supplemented with 2% glucose. The agar is allowed to harden, the plates are inverted and incubated for 48-60 hours at 37° prior to scoring. The zero concentration plates in each series have 0.1ml DMSO, water, or other solvent in place of the mutagen being tested. These plates serve as solvent controls.

3) PREPARATION OF S-9 FRACTION

The S-9 fraction is the 9,000 g supernatant of homogenized rat livers. The livers are obtained from adult Sprague-Dawley rats. The rats are treated with Aroclor 1254 (500 mg/kg) five days prior to sacrifice for induction of high levels of aryl hydrocarbon hydroxylase (AHH) activity. Each S-9 preparation is assayed for protein concentration (mg/ml), AHH activity and promutagen activation of benzo(a)pyrene, 2-aminoanthracene and proflavin. This preparation is able to convert a variety of promutagens to mutagenic status. The activation preparation for 10 ml is prepared as follows:

1.0 ml S-9 fraction

0.2 ml $MgCl_2$ (0.4M)
0.2 ml KCl (0.4M)
0.05 ml glucose-6-phosphate (1M)
0.4 ml NADP (0.1M)
5.0 ml sodium phosphate (0.2M, pH 7.4)
3.15 ml H_2O

4) CONCENTRATION LEVELS

All chemicals are tested at 6 concentration levels (0,.1,1,10, 100,100 μgm/plate) with and without metabolic activation. Liquids are assumed to be of approximately the density of water and diluted appropriately. Chemicals are dissolved in DMSO, water, etc. as is required by individual solubility properties. Subsequent dilutions are carried out in the same solvent. Neither DMSO or water affects the level of spontaneous mutagenesis in this test. The zero dose point for each strain with each chemical is a solvent control (either 100 μl DMSO, water, or other solvent).

5) MUTAGENIC CONTROLS

All strains are tested with a variety of direct and activatable mutagens to demonstrate that the assay is working accurately and efficiently and also to demonstrate that the metabolic activation system is operating.

The mutagens used as positive controls include:

1) FNPA: 4-fluoro-3-nitrophenyl azide; direct-acting against all strains (concentration is 200 μgm/plate).

2) 2NF: 2-nitrofluorene; direct-acting against TA98 and TA1538 (concentration is 10 μgm/plate).

3) NaN_3: Sodium Azide; direct-acting against TA100 and TA1535 (concentration is 1 μgm/plate).

4) 9AA: 9-aminoacridine; direct-acting against TA1537 (concentration is 50 μgm/plate).

Those chemicals which are active against tester strains after metabolic activation include:

1) BP: Benzo(a)pyrene (concentration is 10 μgm/plate).

2) 2AA: 2-aminoanthracine (concentration is 1 μgm/plate).

3) P: Proflavine (concentration is 10 μgm/plate).

The specific solvent used in the experimental protocol provides a solvent mutagenesis level. In addition, the positive controls (described in the previous section) which are diagnostic tests for specific strains, also serve as non-solvent controls for the other strains. For example, sodium azide is a diagnostic positive control for TA100 and TA1535 and can also serve as a negative control for TA98, TA1537 and TA1535. Typical results from mutagenesis experiments performed with control mutagens are given in Table 3. Control data obtained during testing of each submitted chemical will be provided with the corresponding final report.

6) DATA PROVIDED

Experimental results are provided as average revertants per plate for duplicate plates. The zero dose point in each case is a solvent control (DMSO, water, etc.). In addition to results for the specific test compounds, positive and negative control data are also provided as a table (e.g. Table 3).

TABLE 3

CONTROLS FOR SAMPLE: TYPICAL CONTROLS

(Colonies per plate for direct-acting controls)

AGENT	(μgm/plate) CONC.	TA98	TA100	TA1535	TA1537	TA1538
DMSO		20	253	63	8	17
2NF	100	1997	738	87	81	2103
FNPA	200	152	609	181	39	236
NaN_3	1	27	914	860	9	18
9AA	50	18	371	223	1784	17

(Colonies per plate for S-9 activatable controls)

AGENT	(ugm/plate) CONC.	TA98	TA100	TA1535	TA1537	TA1538
2AA	1	39	253	58	12	20
2AA+M	1	945	1443	235	116	583
BP	1	20	267	63	10	11
BP+M	1	174	339	60	99	161
BP	10	28	227	81	23	15
BP+M	10	235	495	77	148	113
P	10	20	321	105	83	26
P+M	10	353	378	116	169	378

KEY:

DMSO: dimethylsulfoxide
2NF: 2-nitrofluorene
FNPA: 4-fluoro-3-nitrophenyl
9AA: 9-aminoacridine
2AA: 2-aminoanthracine
BP: Benzo(a)pyrene
P: Proflavine
M: Microsomes

REFERENCES

1) Ames, B., J. McCann and E. Yamasaki, Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-microsome mutagenicity test, Mut. Res. 31 (1975) p. 347-364.

2) McCann, J., E. Choi, E. Yamasaki and B. Ames, Detection of Carcinogens as Mutagens in the Salmonella/microsome test: Assay of 300 Chemicals, Proc. Nat'l. Academy Sci. 72 (1975) p. 5135-5139.

3) McCann, J. and B. Ames, Detection of Carcinogens as Mutagens in the Salmonella/microsome test: Assay of 300 chemicals: Discussion, Proc. Nat'l. Acad. Sci. 73 (1976) p. 950-954.

4) McKee, R., J.G. Tometsko and A.M. Tometsko, Chemicals which Revert All Commonly Used Salmonella typhimurium Tester Strains, Mut. Res., 67, 183 (1979).

APPENDIX O

Laboratory: Litton Bionetics, Inc.
5516 Nicholson Ln.
Kensington, MD 20795

Submitter: Ms. Sky Benson
Operations Manager
Department of Molecular Toxicology
(301)881-5600

Date of submission: February 24, 1981

Omissions: None

Litton

5516 Nicholson Lane Kensington Maryland 20795 301 881 5600 • Telex 89-8369

February 24, 1981

Stephanie Toney
Genetic Bioassay Branch (MD68)
USEPA, HERL, GTD, GBB
Research Triangle Park, North Carolina 27711

Dear Ms. Toney:

To meet your testing requirements, enclosed is our signed protocol for the Ames/Salmonella Assay. The fixed price to conduct this study is $500.00 per test article. The Standard Contract Provision is enclosed which will apply to the performance of this study unless other arrangements are made with Litton Bionetics, Inc. Also enclosed is a Material Safety Data Sheet which we ask clients to complete with as much information as possible prior to study initiation.

If the enclosed protocol meets with your approval, please complete the "TEST ARTICLE" section, sign in the "APPROVAL OF PROTOCOL" section, and return the signed protocol with your letter of authorization, completed Material Safety Data Sheet, and test article(s). In accordance with Federal Good Laboratory Practice Guidelines, we will be unable to issue a report without the return of the approved protocol.

We look forward to conducting this study for you. Please do not hesitate to contact me if you have any questions.

Sincerely,

Ms. Sky Benson
Operations Manager
Department of Molecular
Toxicology

SB:msr

Enclosures

PROTOCOL NO. 401
EDITION 4

AMES SALMONELLA/MICROSOME PLATE ASSAY

1. OBJECTIVE

The objective of this study is to evaluate a test article for mutagenic activity in a bacterial assay with and without a mammalian S9 activation system.

2. INDICATOR MICROORGANISMS

The following microbial strains of *Salmonella typhimurium* will be routinely used in the plate test:

Strain Designation	Gene Affected	Additional Mutations Repair	LPS	R Factor	Mutation Type Detected
TA-1535	*his* G	Δ *uvr* B	*rfa*	-	Base-pair substitution
TA-1537	*his* C	Δ *uvr* B	*rfa*	-	Frameshift
TA-1538	*his* D	Δ *uvr* B	*rfa*	-	Frameshift
TA-98	*his* D	Δ *uvr* B	*rfa*	pKM101	Frameshift
TA-100	*his* G	Δ *uvr* B	*rfa*	pKM101	Base-pair substitution

Each strain will be cultured in nutrient broth for approximately 16 hours at 37°C (titer of 10^8 to 10^9 cells per milliliter) and used for the mutagenicity test.

The master culture of each strain will be examined for appropriate genetic markers. The date of strain preparation will be documented, and the daily cultures will be examined periodically for appropriate genetic markers.

The selective medium will be Vogel Bonner Medium E with 2% glucose. The overlay agar will consist of 0.6% purified agar with 0.05 mM histidine, 0.05 mM biotin and 0.1M NaCl according to the methods of Ames *et al*. (1975).

3. ACTIVATION SYSTEM

A. S9 Homogenate

A 9,000 x g supernatant prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 (described by Ames et al., 1975) will be purchased from Bionetics Laboratory Products, Litton Bionetics, Inc. and used in this assay.

B. S9 Mix

Components	Concentration per Milliter S9 Mix
NADP (sodium salt)	4 µmoles
D-glucose-6-phosphate	5 µmoles
$MgCl_2$	8 µmoles
KCl	33 µmoles
Sodium phosphate buffer, pH 7.4	100 µmoles
Organ homogenate from rat liver (S9 fraction)	100 µliters

The following stock solutions will be prepared:

0.2M NADP (sodium salt); Millipore filtered
0.2M D-glucose-6-phosphate; Millipore filtered
0.4M $MgCl_2$; sterilize by autoclaving
1.65M KCl; sterilize by autoclaving
0.2M sodium phosphate buffer, pH 7.4; sterilize by autoclaving.

The metabolic activation mixture for each experiment will consist of, for 1 ml:

100 µl S9 fraction
20 µl 0.4M $MgCl_2$
20 µl 1.65M KCl
25 µl 0.2M D-glucose-6-phosphate
20 µl 0.2M NADP (sodium salt)
500 µl 0.2M sodium phosphate buffer, pH 7.4
315 µl sterile distilled water.

AMES SALMONELLA/MICROSOME PLATE ASSAY

FIGURE 1

REVERSE MUTATION ASSAY
[Agar Incorporation Method]

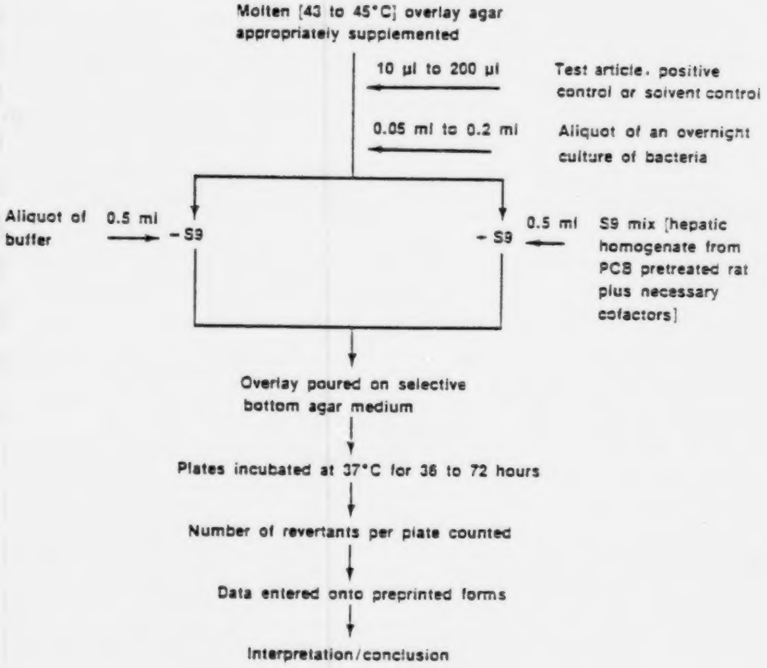
Molten [43 to 45°C] overlay agar appropriately supplemented
10 µl to 200 µl
Test article, positive control or solvent control
0.05 ml to 0.2 ml
Aliquot of an overnight culture of bacteria
Aliquot of buffer
0.5 ml
-S9
+S9
0.5 ml
S9 mix [hepatic homogenate from PCB pretreated rat plus necessary cofactors]
Overlay poured on selective bottom agar medium
Plates incubated at 37°C for 36 to 72 hours
Number of revertants per plate counted
Data entered onto preprinted forms
Interpretation/conclusion

FIGURE 2

COMMERCIAL PLATE ASSAY DATA SHEET

① Chemical Name or Code [1-80] ____________________

Test Dates		Date Completed
M D Y	M D Y	M D Y
__/__/__	__/__/__	__/__/__
[21-26]	[27-32]	[33-38]

② Solvent [1-20] ____________________

Concentration Units ____________ [39-40]

☐ 41   1 = -S9 Mix Only
2 = + S9 Mix Only

S9 Lot# [42-51] ____________

Table# ☐

Client ____________ Date Recvd. ____________ Recvd. By ____________

Physical Description ____________ Assay# ____________

Activation System Employed ____________ Safety# ____________ Tech. ____________

Other Considerations ____________

		Indicator Organisms																	
		TA-1535			TA-1537			TA-1538			TA-98			TA-100					
	Concentration	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
	Solvent																		
	Solvent																		
	Positive																		
	Positive																		
-S9 Mix																			

	Solvent																		
	Solvent																		
	Positive																		
	Positive																		
+ S9 Mix																			

Evaluation/Conclusion ____________ Submitted By ____________

LBI-0184 R 8/80

4. EXPERIMENTAL DESIGN

A. Dosage Selection

In the standard plate assay, six dose levels of the test material, dissolved in a suitable solvent, are evaluated. Routinely, the following doses will be used: 0.01, 0.1, 1.0, 5.0 10.0, 25.0 and 50 µl per plate for liquids and 1.0, 10.0, 100.0, 500.0, 1000.0, 2500.0, 5000.0 and 10,000.0 µg per plate for solids. When the test material exhibits toxicity at the four highest doses with three or more indicator strains, lower doses of the test material will be employed.

If the sponsor specifies doses other than these above doses, the tests are run using the specified doses and these doses will be reported in the interpretation of results and also in the results tables.

B. Mutagenicity Testing

The procedure used is based on the paper published by Ames *et al.*[6] and is performed as follows:

1. Nonactivation Assay

To a Sterile 13 x 100 mm test tube placed in a 43°C water bath the following is added in order:

- 2.00 ml of 0.6% agar containing 0.05 mM histidine and 0.05 mM biotin.
- 0.05 ml of a solution of the test chemical to give the appropriate dose.
- 0.1 ml - 0.2 ml of indicator organism/s.
- 0.50 ml of 0.2M phosphate buffer, pH 7.4

This mixture is swirled gently and then poured into minimal agar plates (see 3B, Media). After the top agar has set, the plates are incubated at 37°C for approximately 2 days. The number of $\underline{his}^+$ revertant colonies growing in the plates is counted and recorded.

2. Activation Assay

The activation assay will be run concurrently with the nonactivation assay. The only difference is the addition of 0.5 ml of S9 mix (see 3C:2, Activation System) to the tubes in place of 0.5 ml of phosphate buffer which is added in nonactivation assays. All other details are similar to the procedure for nonactivation assays.

5. TEST PROCEDURES

A. The following table shows the articles that will be used as positive control agents.

Assay	Chemical	Solvent	Concentration per Plate (μg)	Salmonella Strains
Nonactivation	Sodium azide	Water	10	TA-1535, TA-100
	2-nitrofluorene (NF)	Dimethylsulfoxide	10	TA-1538, TA-98
	9-aminoacridine (9AA)	Ethanol	50	TA-1537
Activation	2-anthramine (ANTH)	Dimethylsulfoxide	2.5	For all strains

B. The solvent used to prepare the stock solution of the test article will be given in the Results Section of the final report All dilutions of the test article will be made using this solvent. The amount of solvent used is equal to the maximum volume used to give the appropriate test dose. The positive and solvent controls will be assayed concurrently along with the test article.

6. EVALUATION CRITERIA

Statistical methods are not currently used, and evaluation will be based on the criteria included in this protocol.

Plate test data will consist of direct revertant colony counts obtained from a set of selective agar plates seeded with populations of mutant cells suspended in a semisolid overlay. Because the test article and the cells are incubated in the overlay for approximately two days and a few cell divisions occur during the incubation period, the test is semiquantitative in nature. Although these features of the assay reduce the quantitation of results, they provide certain advantages not contained in a quantitative suspension test:

- The small number of cell divisions permits potential mutagens to act on replicating DNA, which is often more sensitive than nonreplicating DNA.
- The combined incubation of the test article and the cells in the overlay permits constant exposure of the indicator cells for approximately two days.

AMES SALMONELLA/MICROSOME PLATE ASSAY

6. EVALUATION CRITERIA (Continued)

A. Surviving Populations

Plate test procedures do not permit exact quantitation of the number of cells surviving chemical treatment. At low concentrations of the test article, the surviving population on the treatment plates is essentially the same as that on the negative control plate. At high concentrations, the surviving population is usually reduced by some fraction. Our protocol will normally employ several doses ranging over two or three log concentrations.

B. Dose-Response Phenomena

The demonstration of dose-related increases in mutant counts is an important criterion in establishing mutagenicity. A factor that might modify dose-response results for a mutagen would be the selection of doses that are too low (usually mutagenicity and toxicity are related). If the highest dose is far lower than a toxic concentration, no increases may be observed over the dose range selected. Conversely, if the lowest dose employed is highly cytotoxic, the test article may kill any mutants that are induced, and the test article will not appear to be mutagenic.

C. Control Tests

Positive and negative control assays will be conducted with each experiment and will consist of direct-acting mutagens for nonactivation assays and mutagens that require metabolic biotransformation in activation assays. Negative controls will consist of the test article solvent in the overlay agar together with the other essential components. The negative control plate for each strain will give a reference point to which the test data will be compared. The positive control assay will be conducted to demonstrate that the test systems are functional with known mutagens.

D. Evaluation Criteria for Ames Assay

Because the procedures to be used to evaluate the mutagenicity of the test article are semiquantitative, the criteria to be used to determine positive effects are inherently subjective and are based primarily on a historical data base. Most data sets will be evaluated using the following criteria.

6. EVALUATION CRITERIA (Continued)

D. Evaluation Criteria for Ames Assay

1. Strains TA-1535, TA-1537 and TA-1538

If the solvent control value is within the normal range, a test article that produces a positive dose response over three concentrations with the highest increase equal to three times the solvent control value will be considered to be mutagenic.

2. Strains TA-98 and TA-100

If the solvent control value is within the normal range, a test article that produces a positive dose response over three concentrations with the highest increase equal to twice the solvent control value for TA-98 and TA-100 will be considered to be mutagenic.

3. Pattern

Because TA-1535 and TA-100 are both derived from the same parental strain (G-46) and because TA-1538 and TA-98 are both derived from the same parental strain (D3052), to some extent there is a built-in redundancy in the microbial assay. In general the two strains of a set respond to the same mutagen and such a pattern is sought. Generally, if a strain responds to a mutagen in nonactivation tests, it will do so in activation tests.

4. Reproducibility

If a test article produces a response in a single test that cannot be reproduced in additional runs, the initial positive test data lose significance.

E. Evaluation Criteria for Toxicity

1. Complete Toxicity

When there are no revertants observed on the plate(s) treated with the test compound, the test compound will be defined as toxic to all or any of the indicator strains at that particular dose(s).

6. EVALUATION CRITERIA (Continued)

2. Slight Toxicity

When there are fifty per cent or less number of revertants on the plate(s) treated with the test compound as compared to the solvent control plate(s), the test compound will be defined as slightly toxic to all or any of the indicator strains at that particular dose(s).

The preceding criteria are not absolute, and other extenuating factors may enter into a final evaluation decision. However, these criteria will be applied to the majority of situations and will be presented to aid those individuals not familiar with this procedure. As the data base is increased, the criteria for evaluation can be more firmly established.

F. Relation between Mutagenicity and Carcinogenicity

It must be emphasized that the Ames *Salmonella*/Microsome Plate Assay is not a definitive test for chemical carcinogens. It is recognized, however, that correlative and functional relations have been demonstrated between these two endpoints. The results of comparative tests on 300 chemicals by McCann et al. (1975) show an extremely good correlation between results of microbial mutagenesis tests and *in vivo* rodent carcinogenesis assays.

All evaluations and interpretation of the data to be presented in the final report will be based only on the demonstration, or lack, of mutagenic activity.

7. REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E.: Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian microsome mutagenicity test. Mutat. Res., 31:347-364, 1975.

McCann J., Choi, E., Yamasaki, E. and Ames, B.N.: Detection of carcinogens as mutagens in the *Salmonella*/microsome test: Assay of 300 chemicals. Proc. Nat. Acad. Sci., 72:5135-5139, 1975.

AMES SALMONELLA/MICROSOME PLATE ASSAY

8. RECORDS TO BE MAINTAINED

All raw data, protocol, modifications, test article weight and dispensation records and correspondence between LBI and the Sponsor will be maintained in a central file within the Department of Genetics and Cell Biology. These records will be filed under Departmental assay number and held up to 2 years following submission of the final report to the Sponsor. After 2 years they will be transferred to the LBI Archives for permanent storage.

9. TEST ARTICLE

The test article to be used in this study is ____________________. The identity, strength, purity and composition of each batch and also the stability of the test article under the conditions of administration in the assay, with verifying documentation:

____ will be determined by LBI (at additional cost).
____ will be determined by the Sponsor. Physicochemical data supplied by the Sponsor will be included in the report if requested.

10. PERSONNEL

The Study Director will be D.R. Jagannath, Ph.D. Resumes for personnel are maintained and are available on request.

11. TESTING FACILITIES AND SPONSOR

This study will be conducted at Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, MD 20795. The Sponsor of this study is

12. STUDY DATES

The proposed date of study initiation is 15 days from receipt of test article. The anticipated date of study completion is 3 weeks after study initiation. The anticipated date for submission of the final report to the Sponsor is within 1 month of study completion.

13. CHANGES OR REVISIONS

Any changes or revisions of this approved protocol will be documented, signed by the Study Director, dated and maintained with this protocol. The Sponsor will be notified prior to any change or revision.

AMES SALMONELLA/MICROSOME PLATE ASSAY

14. APPROVAL OF PROTOCOL

Date: ______________________ Sponsor: ______________________

Date: ______________________ Study Director: ______________________

Litton **BIONETICS**

5516 Nicholson Lane Kensington Maryland 20795 [illegible]

Attached is a Material Safety Data Sheet requesting particular information on the test article. This information will be very useful as far as employee health is concerned. For Litton Bionetics, Inc. to comply with Good Laboratory Practice Regulations, the Toxic Substances Control Act, and/or the Occupational Safety and Health Act, we are requesting that this form be completed with as much information as available.

This information will assist our safety programs and expedite the evaluation of the test article.

Litton Bionetics, Inc. emphasizes confidentiality and shall not disclose to others, or use for itself, any information acquired from the Sponsor.

Litton **BIONETICS**

PRODUCT DESIGNATION

MATERIAL SAFETY DATA SHEET

SECTION 1. SOURCE & NOMENCLATURE

SPONSOR'S NAME	EMERGENCY TELEPHONE NO.
ADDRESS (Number, Street, City, State, Zip Code)	
MOLECULAR WEIGHT	CHEMICAL FAMILY
TEST ARTICLE NAME & SYNONYMS	FORMULA

SECTION 2. HAZARDOUS INGREDIENTS

BASIC MATERIAL	APPROXIMATE OR MAXIMUM % WT. OR VOL.	ESTABLISHED OSHA STANDARD	LD_{50}/LC_{50}			
			ORAL	PERCUT.	SPECIES	CONC.

SECTION 3. PHYSICAL DATA

BOILING POINT (°F)	VAPOR PRESSURE (mm Hg)
MELTING POINT (°F)	VAPOR DENSITY (Air = 1)
SPECIFIC GRAVITY (H_2O = 1)	EVAPORATION RATE (________ = 1)
SOLUBILITY IN WATER (Pts/100 Pts H_2O)	VOLATILE %Vol. %Wt.
APPEARANCE AND ODOR	SOLVENTS

SECTION 4. FIRE & EXPLOSION HAZARD DATA

FLASH POINT METHOD USED	FLAMMABLE (EXPLOSIVE) LIMITS	UPPER LOWER
EXTINGUISHING MEDIA		
SPECIAL FIRE FIGHTING PROCEDURES		
UNUSUAL FIRE AND EXPLOSION HAZARDS		

LBI-0082 R 2/79

PRODUCT DESIGNATION

SECTION 5. HEALTH HAZARD DATA (IF AVAILABLE)

TOXIC LEVEL	CARCINOGENIC
PRIMARY ROUTES OF ABSORPTION	SKIN AND EYE IRRITATION
RELEVANT SYMPTOMS OF EXPOSURE	
EFFECTS OF CHRONIC EXPOSURE	
EMERGENCY AND FIRST AID PROCEDURES	

SECTION 6. REACTIVITY DATA

CONDITIONS CONTRIBUTING TO INSTABILITY
CONDITIONS CONTRIBUTING TO HAZARDOUS POLYMERIZATION
INCOMPATIBILITY (Materials to Avoid)
HAZARDOUS DECOMPOSITION PRODUCTS

SECTION 7. SPILL OR LEAK PROCEDURES

STEPS TO BE TAKEN IN CASE TEST ARTICLE IS RELEASED OR SPILLED
WASTE DISPOSAL METHOD

SECTION 8. SPECIAL PROTECTION INFORMATION

VENTILATION REQUIREMENTS LOCAL EXHAUST	PROTECTIVE EQUIPMENT (Specify Types) EYE
MECHANICAL (General)	GLOVES
SPECIAL	RESPIRATOR
OTHER PROTECTIVE EQUIPMENT	

SECTION 9. SPECIAL PRECAUTIONS

PRECAUTIONS TO BE TAKEN IN HANDLING AND STORAGE
OTHER PRECAUTIONS

Signature	Date	Address

APPENDIX P

Laboratory: Meloy Laboratories, Inc.
6715 Electronic Dr.
Springfield, VA 22151

Submitter: Dr. Carol L. Richardson
Principal Scientist
(703)642-4868

Date of submission: December 9, 1980

Omissions: None

MELOY LABORATORIES, INC.
A Wholly Owned Subsidiary of REVLON, INC
6715 Electronic Drive, Springfield, Virginia 22151 • (703) 354-2600
Telex 899-440

December 9, 1980

Dr. S. H. Toney
Genetic Bioassay Branch
USEPA, HERL, GTD, GBB (MD-68)
Research Triangle Park, N.C. 27711

Dear Dr. Toney:

Per your request of November 26, 1980, I have enclosed a copy of our current Ames testing protocol. Currently, we are making additions to these basic procedures in order to be in compliance with GLP in the very near future. As soon as these additional procedures are completed, I will send you a copy, if deemed necessary.

As you may know, Dr. Diana Anderson has recently been involved in coordinating an international study to test for genetic drift among Ames strains. A great deal of time and effort was expended in developing the standardized protocol for this study which may be helpful to you. If you do not already have a copy of it, I'm sure Dr. Anderson would be happy to send you one. Her address is ICI Limited, Central Toxicology Laboratory, Olderley Park, Macclesfield, Cheshire SK104TJ U.K.

If I can provide further information, please do not hesitate to call me at (703) 642-4868.

Sincerely yours,

MELOY LABORATORIES, INC.

Carol L. Richardson

C. L. Richardson, Ph.D.
Principal Scientist

CLR:rrr

Enclosure: Ames protocols

Diagnostics • Life Sciences • Instruments and Systems
REVLON HEALTH CARE GROUP

Safety Precautions for Working With Carcinogens

Storage, weighing, and disposal procedures

All carcinogens will be kept in the storage cabinet farthest from the door. The cabinet will be clearly marked CAUTION--CARCINOGENS. Upon receipt of a carcinogenic (or potentially carcinogenic substance), it will be logged in the gray bound notebook; the total amount, date and initials of the receiving person will be entered. Each time a carcinogen is used the amount will be deducted in the log book. In this way a running inventory on all carcinogens is maintained.

The primary container is kept in a screw cap, metal secondary container. Both will be labelled. The secondary container can be carried safely within the lab. The primary container is removed only under a hood and carcinogens are weighed on the M-5 balance in the glove box. Following the weighing procedure, the primary container and the vessel into which the weighed compound has been placed are passed into the Class II Type II hood. These are placed into secondary containers or diluted prior to removal from the hood.

Upon completion of work with a given solution, it is to be deactivated if possible and appropriately disposed of. Disposal of all dry contaminated materials (pipettes, vials, tubes, etc) will be handled by double-bagging and placing in waste barrels which will be labelled with the compound, maximum amount, and initials of the depositor in addition to a biohazard label. The Safety Officer will be responsible for removal of these from the premises.

Liquids containing carcinogens will be poured into metal screw-cap containers which are 2/3 full of vermiculite-charcoal. Solvent should be allowed to evaporate off under the hood. Containers are then filled to the top with charcoal, sealed, bagged and disposed of as dry waste. If the compound can be readily deactivated, this method of disposal is preferred. Deactivation methods include oxidation with sodium hypochlorite, treatment with 5% KOH, 6N HCl, or digestion in chromic acid.

Clean-up of Spills

Solutions containing carcinogens or dry solids which have been spilled on a non-disposable surface should be de-activated *in situ* if possible. Solutions should be absorbed onto paper. Solids are dissolved in a minimum of appropriate solvent and then handled as a solution. The contaminated surface is then washed at least twice with acetone and followed by hot alkaline detergent (Alconox) and water.

In an emergency situation, the emergency switch is tripped which alters the air intake into the laboratory; entrance of other individuals is prevented. If the spill involves a dry powder, the individual should put on a respirator and then proceed with the clean-up. If procedures with secondary containers are adhered to, this type of emergency should never occur. Following the clean-up, the individual shall shower out.

Standard Safety Measures for Carcinogen Work

1. Absolutely no smoking, eating, drinking or food storage will be permitted in the laboratory.

2. Mouth pipetting is not permitted. Various types of automatic pipetting devices are available.

3. Immediately after completion of procedures involving carcinogens, the hands sould be washed in warm water with soap.

4. If Category III carcinogens have been used in the laboratory, individuals working with them shall shower out prior to leaving. Category III carcinogens include: aflatoxins, 4-aminobiphenyl, benzidine, Bis-(chloromethyl) ether, 3,3'dichlorobenzidine, 3,3'dimethoxybenzidine, 3,3'dimethylbenzidine, 2-naphthylamine, 4-nitrobiphenyl.

Protective Clothing

All persons handling carcinogens must wear protective clothing, consisting of a paper lab coat to be worn over the standard cloth lab coat and disposable gloves. The paper lab coats are disposed of at the end of each working day. Persons should *never* take contaminated clothing home.

Paper lab coats and used disposable gloves can be placed in a combustible plastic or paper bag labelled " Carcinogenic" or "Carcinogen" and burned or placed in the waste barrel with other carcinogenic waste. For all work with carcinogenic substances in DMSO or other solvents, 15 mil latex gloves provide adequate protection. Triple Latex Conform gloves satisfy the safety requirements and still allow sufficient flexibility. However, they should not be worn longer than 4 hours. Those using neoprene gloves must wash the gloves, while still wearing them in hot alkaline detergent solution (Alconox). This should effectively decontaminate the gloves.

Persons should be reminded frequently that while wearing contaminated gloves they should not touch objects or surfaces to be kept free of contamination (e.g. door knobs, drawer pulls, telephones, record books, etc.).

Limited Access

The work and storage areas where carcinogens are present should be identified with conspicuous signs stating:

CAUTION --CHEMICAL CARCINOGENS
Authorized personnel only

Such signs will be placed at all entrances to these areas. The entry to the mutagenesis/carcinogenesis laboratory will be by key only. The key will be available from designated authorized persons. A list of authorized persons will be posted on the door. The janitorial staff will not be allowed free access to this laboratory.

Equipment repair will require the serviceman to be informed of the possible contamination, and made to wear a lab coat and gloves. When servicing the balance is necessary the balance should be left in the hood. The tools used to service the balance should be dipped in an appropriate solvent which can be effectively burned or saturated with a solution which inactivates the carcinogen. After a solvent rinse, the tools should be rinsed in bleach or hot alkaline detergent. Where this is not possible, a set of tools may be reserved for the purpose of servicing the contaminated balance only, and kept with the balance.

References:

1. Guidelines for the Laboratory Use of Chemical Substances Posing a Potential Occupational Carcinogenic Risk; Laboratory Chemical Carcinogen Safety Standards Subcommittee of the DHEW Committee to Coordinate Toxicology and Related Programs, Revised Draft, June 5, 1979.

2. Suspected Carcinogens, A subfile of the Registry of Toxic Effects of Chemical Substances, NIOSH, DHEW, 2nd Edition, 1976.

3. Toxic Substances Control I Law and Regulations and II Proceedings of the Second Annual Toxic Substances Control Conference, 1977. M. L. Miller, ed. Government Institutes, Inc.

4. Handbook of Mutagenicity Test Procedures; E. J. Kilbey, M. Legator, W. Nichols, and C. Ramel, Elsevier Scientific N.Y. 1977.

5. National Cancer Institute Safety Standards for Research Involving Chemical Carcinogens, Office of Research Safety, DHEWPublication No. (NIH) 76-900.

6. Handling of Carcinogens and Hazardous Compounds, Calbiochem Technical Manual.

7. The Permeability of Laboratory Gloves to Selected Solvents. E. B. Sansone and Y. B. Tewari, Am. Indust. Hygeine Assoc. J. 39:169-174. 1978.

(Note: Dr. Richardson has a copy of all of the above references.)

Procedure for Weighing Carcinogens

1. Put on triple latex gloves; turn on hood & glove box lights.

2. Place the following items in the right glove box pass-through:

   trash bag
   countertop pad
   disposable spatulas; turn on glove box lights

3. Use disposable tubes, unwrap, under hood, add 1 ml DMSO to each & label. Place in glove box through left pass-through. DMSO is a highly permeable solvent & will carry almost any chemical with it through the skin. Measure out carefully over a countertop pad. In the event that it is spilled, take several thicknesses of paper toweling or gauze and wipe up immediately, discard all 3 layers of gloves that have come in contact with this solvent and put on clean gloves.

4. Put on disposable gown over lab coat.

5. Check that there are sufficient weigh papers and gauze pads & solvent (acetone) in glove box for clean up.

6. Remove carcinogen(s) to be weighed from storage cabinet. Remove outermost "contaminated glove" & put on new one.

7. Put gloved hands inside black neoprene gloves.

8. Take items from right pass-through. Spread countertop pad next to balance, place all other items at back of hood out of the way.

9. Using large forceps, remove weigh paper from petri dish & place on balance pan. (Do not tare at this time). Leave balance door open.

10. Remove carcinogen bottle from secondary container; remove cap of carcinogen bottle and place inverted on countertop pad.

11. Using spatula, remove small amount of compound & place on weigh paper in balance compartment. Put spatula into trash bag immediately & cap carcinogen bottle.

12. Close balance compartment door. Set balance on partial release (bottom knob is released to the right). Generally, dials needs to be set on .07 or .08 until a number is seen on the dial. Then set to full release (bottom knob all the way to the left).

13. Remove hand from the neoprene glove & record weight on the pad on the outside of the glove box.

14. Put balance on arrest (bottom knob in vertical position).

15. With forceps, remove weigh paper & put chemical into tube containing 1 ml DMSO.

16. Replace weigh paper on balance pan & determine tare. Record on pad below total weight. Subtract tare to get total weight. Return balance to arrest position.

17. Return carcinogen bottle to secondary container & place in right glove box pass-through after wiping off outside of secondary container.

18. Fold up countertop pad & wipe down balance below pan & area around balance. Put countertop pad and towels in trash bag. Close trash bag & place in pass-through.

19. Set rack & tube containing carcinogen solution in left hood pass-through.

20. Remove neoprene gloves, record amount of carcinogen weighed out in log book.

21. Remove carcinogen in secondary container from pass-through & return to storage cabinet (or refrigerator).

22. Remove trash bag to double bag or place in waste can after taping securely. Record carcinogen & maximum amount on list on top of waste can; date & initial same.

23. Remove outermost pair of gloves.

24. Remove weighed out sample to hood and make appropriate dilutions.

25. Remove second outer pair of gloves, then gown & final pair of gloves. Wash hands before leaving lab.

Ames Assay - Media and Solutions

VBE Agar Plates

1. VBE Salts 25x Stock

   To 700 ml distilled H_2O add: 10 gm $MgSO_4 \cdot 7H_2O$ (use 4.89 gm if anhydrous), stir until dissolved.

   Add 100 gm Citric acid, stir until dissolved.

   655.7 gm $K_2HPO_4 \cdot 3H_2O$ (Dibasic Potassium phosphate; use 500 gm if anhydrous). Stir until completely dissolved, may need to heat to 45°C.

   Add 175 gm $NaNH_4\ HPO_4 \cdot 4H_2O$ (sodium ammonium phosphate). Stir until dissolved, Q.S. to 2 liters.

   Salts must be added slowly with continuous stirring in the order given above. Autoclave, cool and store at room temperature.

2. To make plates:

   a. Weigh 30 gm Noble or purified agar and dilute to 1 liter with dH_2O, heat to dissolve.

   b. Mix 80 ml VBC 25x stock with 920 ml dH_2O

   Measure into 500 ml bottles: 100 ml Noble agar
   100 ml diluted VBC

   Autoclave, cool to 55°C, add 10 ml sterile glucose solution (40% glucose), swirl gently and pour plates.

Minimal Top Agar

Weigh out: 12 gm Noble's or purified agar
10 gm NaCl
Dilute to 2 liters
Heat to dissolve
Dispense in 250 ml bottles (100 ml/bottle)
Autoclave and cool
Heat in boiling water bath, cool to 55°C and add Histidine-Biotin prior to use (10 ml histidine-biotin/100 ml agar)

Histidine-Biotin Solution

Weigh out 20.96 mg L-histidine$\cdot HCl \cdot H_2O$
Add 150 ml dH_2O (adjust the pH with KOHin order to solubilize)
Add 24.4 mg biotin
Q.S. to 200 ml
Filter sterilize
Store at 4°C

Nutrient Broth

Weigh out 8 gm dehydrated nutrient broth/1 liter dH_2O, add 5 gm NaCl
Dispense in tubes (10 ml/tube)
Autoclave
Store at 4°C after sterility check

Nutrient Agar Plates

Weight out 18.6 gm dehydrated nutrient agar, add 600 ml dH_2O, heat to dissolve, autoclave, cool and pour

$MgCl_2$-KCl

8.13 gm $MgCl_2$
12.3 gm KCl
Q.S. to 100 ml with dH_2O
Autoclave
Store at room temperature

Sodium Phosphate

2.84 gm Na_2HPO_4 (Dibasic)
Q.S. to 100 ml with dH_2O
Autoclave
Store at room temperature

Glucose-6-Phosphate

2.82 gm/10 ml dH_2O, filter sterilize, dispense in 0.3 ml aliquots and store frozen. (Do not freeze-thaw more than once).

NADP

0.765 gm/10 ml dH_2O, filter sterilize, dispense in 600 µl aliquots, store frozen. (Do not freeze-thaw more than once).

67 mM Phosphate Buffer

Monobasic stock 0.804 gm NaH_2PO_4/100 ml dH_2O
Dibasic stock 0.951 gm Na_2HPO_4/100 ml dH_2O
To 80 ml dibasic stock, add sufficient monobasic to adjust pH to 7.2
Autoclave
Store at room temperature

Sterile DMSO

For use as solvent, filter sterilize into 50 ml sterile bottles.

Sterile dH_2O

Dispense 50 ml/bottle
Autoclave

For 50 Plates

50 VBC plates
150 ml minimal top agar
15 ml histidine-biotin
2 tubes nutrient broth
2 nutrient agar plates

For 12.5 ml Activating System

0.067 ml glucose-6-P
0.533 ml NADP
0.267 ml $MgCl_2$-KCl
6.667 ml Ha_2HPO_4
3.1 ml dH_2O
Plus 1.25 ml dH_2O, 1.25 ml S-9 (adjusted depending on S-9 activity)

Q.C. of VBE Plates

1. Randomly collect 15 VBE plates as soon as they arrive from media. Store remaining plates in an area where they will not be used until the Q.C. has been completed.
2. Preincubate the 15 VBE plates overnight at 37°C for 24 hrs. to detect any contamination in the media.
3. Prepare stocks of TA-100 and TA-1538 using nutrient broth with 0.5% NaCl. Frozen stocks (-80°C) should be used to inoculate the nutrient broth. An OD560 of .700 or greater should be obtained before use in the assay.
4. Weigh out samples of N-Methyl-N^1-Nitro-N-Nitrosoquanidine (MNNG) and 2-Nitrofluorene (2-NF) in DMSO. Dilute compounds to concentrations of 50 ug/ml and 5 ug/ml, respectively.
5. Set-up Q.C. assay according to protocol on following page. Incubate plates at 37° for 48 hrs. Count revertants.
6. After completion of assay (72 hrs.) check the remaining plates for any contamination that may have been overlooked. If none are found, plates can be used for assays. Any contaminated plates should be sent to decon and discarded.

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Q.C. of VBE Plates

Data Sheet

Date:____________________

Initials:____________________

Bacteria: OD560

TA-100 F/S____________________
TA-1538 F/S____________________

Top agar: 1 bottle with 10 mls histidine-biotin solution.

MNNG:	Total Wt.	2-NF	Total Wt.
-__________	Tare	-__________	Tare
	Net Wt.		Net Wt.
+	ml DMSO	+	ml DMSO
diluted __________	for 50 ug/ml	diluted __________	for 5 ug/ml

Plate #	(2.0 mls) Agar	(100 ul) mutagen	(100 ul) bacteria	Revertants/ Plate	Mean ± Std. Dev.
1-3	+	MNNG	TA-100		
4-6	+	DMSO	TA-100		
7-9	+	2-NF	TA-1538		
10-12	+	DMSO	TA-1538		
13	+	MNNG	0		
14	+	2-NF	0		
15	+	DMSO	0		

For comparison to previous results, reversions for TA-100 should be greater than 2,000 colonies/plate and TA-1538 should be greater than 100 colonies/plate

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Protocol for Preparation of Arochlor Solution

Weigh empty sterile brown glass bottle. Put on gloves. Under hood, with counter-top pad on surface, decant Arochlor into sterile bottle using a sterile pipette as a decanting rod, being very careful not to drip. Close bottles, place each in secondary containers. Discard pipette and counter-top pad into biohazard bag as well as gloves.

Weigh bottle (which has just had Arochlor decanted into it.) Be sure to have bottle in secondary container when carrying it to and from the balance. Return Arochlor to hood. Calculate the amount of corn oil to be added as follows: Determine the amount of Arochlor decanted (weight of bottle containing Arochlor less tare). Volume of sterile corn oil to be added =

$$(\text{wt. of Arochlor} \times 5) - \frac{\text{wt. of Arochlor (gm)}}{1.57\ \text{gm/ml}}$$

This gives a final concentration of 200 mg/ml.

The sterile corn oil is added with a sterile 10 ml pipette under the hood, aseptically.

Protocol for S-9 Preparations

1. Turn on J21C centrifuge, set temperature to 4°C.
2. Preweigh four 100 ml sterile tri-pour beakers which contain 5 ml 0.15 M KCl each.
3. Clean polytron with ethanol.
4. Livers are removed aseptically under a hood, and placed in the pre-weighed beakers on ice.
5. Weigh beakers with livers, compute total liver weight, and multiply by 3 to determine the total amount of KCl to be added.
6. In the Class II type 2 safety cabinet, mince the livers in one of the beakers with sterile scissors, transfer to a 400 ml sterile tri-pour, add approx. 50 ml KCl, homogenize using the polytron until a uniform consistency is obtained, then pour contents into a sterile centrifuge bottle.
7. Repeat step 6 for the three remaining beakers of livers, making sure the total volume of KCl used is 3 times the total liver weight. Use only 3 centrifuge bottles for homogenates.
8. The centrifuge bottles are wiped clean with alcohol-soaked gauze and placed immediately in an ice bucket outside the hood.
9. Clean the polytron with alcohol, and remove any connective tissue with forceps, clean the hood.
10. The 3 centrifuge bottles containing homogenates are balanced with 3 bottles containing water. ONLY the water bottles are opened outside the hood.
11. Homogenates are centrifuged in the JA-14 rotor (J21C centrifuge) for 10 minutes at 8,500 rpm. Be sure the centrifuge is at 4°C.
12. Following centrifugation, the bottles are placed on ice and returned to the hood.
13. The supernatants are pooled in a sterile 1 liter tri-pour, then swirled gently.
14. Using a sterile 10 ml pipette and quantapettor (or other remote pipetting device), 2 ml of supernatant is dispensed into sterile dram vials, and kept on ice until all vials are filled.
15. Vials are returned to the cartons, labeled, and placed in the Revco.

16. At least 3 samples are sterility-checked in thioglycollate broth.

It is extremely important that the livers and homogenates are kept at 4°C through the entire procedure to prevent enzyme degradation.

STRICT ADHERENCE TO SAFETY PRECAUTIONS IS ESSENTIAL.

Any time the livers or homogenates are in open vessels, they should be handled only with gloves under a Type II Class II hood. All tri-pour beakers, pipettes, countertop pads, gloves, etc. should be placed in a bag under the hood after use. They should be removed into a second bag when removed from the hood, and disposed of as carcinogenic waste or incinerated. Individuals working with this preparation should be familiar with the safety precautions for working with carcinogens.

To Prepare Frozen Stocks

1. Inoculate 10 ml of nutrient broth (or other media suitable for the organism - - i.e. LBE for *E. coli* BR513)
   Use either a well-characterized culture from a known frozen-stock or a single colony isolate.
2. Grow isolate overnight at 37° with shaking. Toxin producing *S. aureus* stocks should be grown to OD_{560} between 0.1 to 0.4.
3. Add 875 µl of sterile DMSO per 10 ml of broth, vortex well, aliquot into vials.
4. Store vials at -80°C in Revco.
5. Give to Nancy for streak plate, gram stain, (+ API if applicable). Q.C. for activity of *S. aureus* stocks by coagulase and toxin production by Ouchterlony plate.
6. Record all subcultures and Q.C. info in permanent log.

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To Check Tester Strains

1. Use a broth culture obtained from a single colony isolate or prepare a suspension of a pure culture in sterile phosphate buffer.

2. Using a loop streak:

   minimal agar (VBC) plate
   minimal agar with 0.1 ml histidine-biotin solution spread on surface
   nutrient agar--irradiate half this plate with UV light at a height of 33 cm for 6 sec. (TA 1533, 1537, 1538) or 8 sec. (TA 98, TA 100). Leave half the plate covered.

3. Using a swab prepare:

   nutrient agar or Mueller-Hinton as you would for a sensitivity
   place an ampicillin disc on the surface with sterile forceps and a blank disc to which is added 0.1 ml sterile crystal violet solution (1.o mg/ml).

4. Plates are incubated 24-48 hours--results should be as follows:

TA	98	100	1535	1537	1538
min.	-	-	-	-	-
min. + his. Bio.	+	+	+	+	+
Crystal violet	S	S	S	S	S
Ampicillin	R	R	S	S	S
UV	Growth only on unirradiated slide.				

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STANDARD
PRELIMINARY TOXICITY TEST

Protocol:

1. Solubilize a test compound in a given solvent (dH_2O or DMSO) at 30 mg/ml concentration.
2. Prepare half-log dilutions (factors of 3 and 3 1/3) for 10 concentrations and one 0 μg/ml.
   NOTE: If solubility limits of chemical are <30 mg/ml, attempt the next lower dilution, etc.
3. Add 100λ of each dilution to 4.8 ml nutrient broth, 0.5% NaCl; then add 100λ of an overnight broth culture of TA 1538 to each sample.
4. Prepare 2 sterility controls:
   a) 4.9 mls midea/100λ solvent
   b) 4.9 mls media/100λ highest dilution
5. Incubate tubes for 24 hours at 37°C on shaker.
6. Measure OD_{560} values.
7. Calculate 8/0 G/G_o*
8. Determine $\sim I_{50}$ dose for 24 hours.

*G = Growth at 24 hours
G_o = max. growth at 24 hours for 0 μg/ml.

NOTE: The level of test agent which produces an approximate 50% reduction in growth rate is the highest level used in the plate incorporation assay.

Tube Example: plate = tube

	1	2	3	4	5	6	7	8	9	10	11	12
mg/ml	10	3	1	0.3	0.1	0.03	0.01	0.003	0.001	0	0	10
mg/plate	1	0.3	0.1	0.03	0.01	0.003	0.001	0.0003	0.0001	0	0	

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Procedure for the Spot Test

1. Grow up tester strain as described.
2. To 2.0 ml of minimal top agar with histidine and biotin, add 0.1 ml bacteria, and in half the tubes, add 0.5 ml of activating system.
3. Mix and then pour the liquid top agar onto minimal agar plates.
4. After the agar has solidified, place a small sterile, filter paper disc in the center of the plate.
5. Immediately add 0.1 ml of the chemical to be tested, at a concentration of 5 mg/ml/
6. Incubate the plates at 37°C for 48 hrs.
7. After 48 hrs check for stimulation or inhibition around the disc. Compare with a control compound for the given strain.

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Procedure for Running the Plate Incorporation Assay

1. Grow up strains as described previously.

2. Spin down the cells and resuspend in 1X VBC, or 67 mM phosphate buffer.

3. Melt the top agar (2.0 ml for each plate) in boiling water bath. Once the top agar is melted down, keep it in 56° water bath at all times. (If the top agar is allowed to cool down even the slightest bit, it becomes very grainy). The top agar cannot be used if allowed to resolidify.

4. Prepare the activating system (A.S.) and dilutions of the S-9; be sure to keep both of the solutions on ice at all times.

For 50 Plates (20 containing A.S.)	For 150 Plates	
.07 ml	0.2 ml	Glucose-6-Phosphate
0.5 ml	1.6 ml	NADP
0.3 ml	0.8 ml	$MgCl_2$-KCl
6.7 ml	20.0 ml	Na_2HPO_4
3.1 ml	9.4 ml	dH_2O
2.5 ml	7.5 ml	S-9 diluted with H_2O to proper concentration

5. When the top agar has been melted, add to it the histidine-biotin solution (10 ml/100 ml agar).

6. Filter sterilize the activating system using a 0.45 μ Nalgene filter.

7. The chemical to be tested and appropriate controls should be weighed in the glove box and put into either H_2O or DMSO. It is imperative that proper safety precautions are adhered to. Dilutions are prepared in the hood.

8. When the solutions, chemical, bacterial suspensions, top agar and petri plates are ready, the assay can be started. Be sure to check the temperature of the heating block before proceeding.

9. Nine tubes are usually the maximum that can be placed in the hot block at once and not deactivate the generating system. Once the activating system has been added to the tubes, they should be poured immediately to prevent deactivating the enzymes in microsomal fraction.

10. Add the different components of the assay in the order indicated on the protocol.

11. Gently mix the contents of the tube on the Vortex Genie before pouring each plate.

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12. After pouring each plate, swirl gentlyand allow the top agar to solidify.

13. After all plates have been poured and allowed to solidify, they are stacked on trays, labeled and incubated at 37° for at least 48 hours.

14. Plates can be counted automatically or individually using a manual colony counter and data recorded in ink in a bound notebook.

15. All tubes, pipettes, petri plates, and bottles used in assay should be disposed of as described in the section of Safety Precautions.

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POSITIVE CONTROL MUTAGENS FOR EACH STRAIN

Bacterial Strain	Without Microsomal Enzymes		With Microsomal Enzymes	
	Mutagen	µg/plate	Mutagen	µg/plate
Salmonella typhimurium				
TA 98	2-Nitrofluorene	0.5	Benzo(a)pyrene	10
TA 100	MNNG*	0.75	Benzo(a)pyrene	10
TA 1535	MNNG	0.75	2-Aminoanthracene	3
TA 1537	9-Aminoacridine	30.0	Benzo(a)pyrene	10
TA 1538	2-Nitrofluorene	0.5	Benzo(a)pyrene	10

*MNNG=N-methyl-N-nitro-N-nitrosoguanidine

Plate Incorporation Assays

Protocol for One Bacterial Strain

Plate #	Agar 2.0 ml	Chem. 0.1 ml	Bact. 0.1 ml	Activating System or Buffer 0.5 ml[1]
1-3	2.0 ml	Diln. #1	0.1 ml	Buffer
4-6	"	" #2	"	"
7-9	"	" #3	"	"
10-12	"	" #4	"	"
13-15	"	" #5	"	"
16-18	2.0 ml	Diln. #1	0.1 ml	A.S.[2]
19-21	"	" #2	"	"
22-24	"	" #3	"	"
25-27	"	" #4	"	"
28-30	"	" #5	"	"
Spontaneous Revertants				
31-33	2.0 ml	Solvent	0.1 ml	Buffer
34-36	"	"	"	A.S.
Positive Controls				
37-39	2.0 ml	Mutagen	0.1 ml	Buffer
40-42	"	"	"	A.S.
Sterility Controls				
43	2.0 ml	Solvent	-	Buffer
44	"	Diln. #1	-	"
45	"	" #2	-	"
46	"	" #3	-	"
47	"	" #4	-	"
48	"	" #5	-	"
49	"	Mutagen	-	"
50	"	-	-	"
51	"	-	-	A.S.

[1] The buffer is 67 mM phosphate, pH 7.2.

[2] Activating system.

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Meloy Laboratories, Inc.

MUTAGENICITY REPORT

Compound:______________________ Report to:______________________

Source:______________________ ______________________

Tester Strain	µg/Plate	Revertants/Plate Without S9	 With S9
TA 98			
Spontaneous Solvent_____			
Positive Controls			
TA 100			
Spontaneous Solvent_____			
Positive Controls			

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Tester Strain	µg/Plate	Revertants/Plate Without S9	With S9
TA 1535			
Spontaneous Solvent_____			
Positive Controls			
TA 1537			
Spontaneous Solvent_____			
Positive Controls			
TA 1538			
Spontaneous Solvent_____			
Positive Controls			

Bacterial Toxicity Data:

Conclusions/Comments:

Standard Ames Test, procedure according to: Ames, B. N. et al. Mutation Res. 31:347-364 (1975).

Investigator:____________________

Approved:____________________

Date:____________________

References for Procedures:

1. Ames, B. N., McCann, J., Yamasaki, E. 1975 Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. Mutation Research 31: 347-364.

2. McCann, J., Choi, E., Yamasaki, E. and Ames, B. N. 1975 Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals. Proc. Natl. Acad. Sci. 72: 5135-5139.

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S-9 DATASHEET

I. Lot Number: ____________________

Animal Type: Male S-D rats/180-210g

Animal Arrival Date: ____________________

Received From: ____________________

Induced On: ____________________

Induced With: Arochlor 1254, 0.5 cc of 200 mg/ml per rat

Date Sacrificed: ____________________

Date Homogenized: ____________________

Homogenized By: ____________________

Volume Produced: ____________________

Dispensed Into: ____________________ vials @ 2 mls each

____________________ vials @ 5 mls each

II. Sterility Check (3 thioglycollate samples), take samples at 100 ml intervals.

1. 1-100 mls: ____________________
2. 200-300 mls: ____________________
3. 400-500 mls: ____________________

Liver Weights:

Total
Beaker- ______ - ______ - ______ - ______ - ______ - ______ - ______ - ______
Net

Total Liver Weight ______________ x 3 = ______________ Total ml of KCl

-______________ ml KCl in Beakers

-______________ 50 ml KCl/Beaker

______________ Additional Volume KCl

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Lowry
S-9 Lot#________________
Tested By________________
Date________________

Tube #	dH_2O	BSA 1 mg/ml	S-9 µl 1:50	S-9 µl 1:100	OD_{700}
1	100	0			
2	100	0			
3	90	10			
4	90	10			
5	80	20			
6	80	20			
7	70	30			
8	70	30			
9	60	40			
10	60	40			
11	50	50			
12	50	50			
13	50		50		
14	50		50		
15	25		75		
16	25		75		
17	50			50	
18	50			50	

Mean S-9 mg/ml = __________

BSA Curve
Slope = ________________
Reciprocal = ________________

ODxReciprocal µg/tube	x $\frac{1}{\mu l}$=mg/ml	x diln.	mg/ml/sample
	1/50	50	
	1/50	50	
	1/75	50	
	1/75	50	
	1/50	100	
	1/50	100	

PROCEDURE:

Add 1.0 ml Soln. C. to each tube and vortex.
Let stand at room temp. 20 minutes.
Add 100 µl Soln. D to each tube and vortex immediately after addition.
Let stand 30 minutes to 2 hours at room temperature and read OD_{700}.

REAGENTS:

A = 2g NaOH, 100g Na_2CO_3, 0.1g NaK tartrate, Q.S. to 500 ml with dH_2O.
B = 0.5g $CuSO_4 \cdot 5H_2O$, Q.S. to 100 ml with dH_2O.
C = Mix 2 parts B with 100 parts A (i.e., 1 ml B + 50 ml A). Prepare fresh.
D = Folin Phenol Reagent diluted 1:1 with dH_2O.

BOTTOM OF IMAGE AREA OUTSIDE DIMENSION FOR TABLES AND ILLUSTRATIONS

AHH

S-9 Lot#______________

Tested By______________

Date__________________

Tube #	Diln.	Buffer-Cofactor 380λ	Enzyme 200λ	"O" Time Acetone 500λ	BP* 40λ	30' at 37°C	Ice Bath Acetone 500λ	Hexane 1.6 ml	1N NAOH 1.5 ml	Relative Fluorescence Intensity
1	1:10	↓	↓	-	↓	↓	↓	↓	↓	
2	1:10	↓	↓	-	↓	↓	↓	↓	↓	
3	1:20	↓	↓	-	↓	↓	↓	↓	↓	
4	1:20	↓	↓	-	↓	↓	↓	↓	↓	
5	0	↓	Tris	-	↓	↓	↓	↓	↓	
6	0	↓	↓	-	↓	↓	↓	↓	↓	
7	1:10	↓	↓	↓	↓	↓	-	↓	↓	
8	1:20	↓	↓	↓	↓	↓	-	↓	↓	
9	0	↓	Tris	↓	↓	↓	-	↓	↓	
10	Standard 200 ng/ml		3-OH BP	—	-	-				

Calculations:

Tube #	RFI	S.W.	RFI/SW	ng	Prot. Conc.[1] ng/ml	Protein[2] mg/200 µl	ngx diln. / 30 x 269.3 x Prot. Conc[2] nmoles 3-OH BP/min/mg Prot.
1							
2							
3							
4							
5							
6							
7			These values must be <.5 for				
8			an acceptable assay.				
9							
10			This value should be 21 on S.W. of 2.0.				

[2] From Lowry

[2] Use Prqtein Conc. = mg/200 µl

S-9 CONTROL DATA PROTOCOL

Plates:______________________ Solvent:__________________ S-9 Lot#____________

Top Agar:____________________ Buffer:______________________________________

BP:__________________________ Total Wt. | 2AA:_________________________ Total Wt.

10 µg/-______________________ Tare | 3 µg/-_______________________ Tare

plate________________________ Net Wt. | plate________________________ Net Wt.

+____________________________ ml DMSO | +____________________________ ml DMSO

Diluted______________________ for 100 µg/ml | Diluted______________________ for 30 µg/ml

Bacteria:	OD560	CV	UV	AMP	Stock
TA 98					F/S-
TA 100					F/S-
TA 1535					F/S-
TA 1537					F/S-
TA 1538					F/S-

Activating System:

G-6-P - 70 µl
NADP - 500 µl
Na_2PO_4 - 6.7 mls

$MgCl_2$-KCl - 300 µl
dH_2O - 4.3 mls
S-9 - 1.3 mls

filter sterilize w/
0.45 Nalgene

S-9 BIOASSAY PROTOCOL

Plates:______________________ Solvent:__________________ S-9 Lot#____________

Top Agar:____________________ Buffer:______________________________________

BP:__________________________ Total Wt. | DMBA:_________________________ Total Wt.

10 µg/-______________________ Tare | 10 µg/-______________________ Tare

plate________________________ Net Wt. | plate________________________ Net Wt.

+____________________________ ml DMSO | +____________________________ ml DMSO

Diluted______________________ for 100 µg/ml | Diluted______________________ for 100 µg,

Bacteria:	OD560	CV	UV	AMP	Stock
TA 98					F/S-
TA 100					F/S-

Generating System (GS):

G-6-P - 420 µl
NADP - 3.0 mls
Na_2HPO_4 - 40.2 mls

$MgCl_2$-KCl- 1.8 mls
dH_2O - 25.8 mls

S-9 Concentrations:

	S-9 1/ml	µl/plate	S-9	G.S.	DH2O
	0	0	0 µl	8.0 mls	2.0 mls
	20	10	200 µl	8.0 mls	1.8 mls
	40	20	400 µl	8.0 mls	1.6 mls
Recommended Range	60	30	600 µl	8.0 mls	1.4 mls
	80	40	800 µl	8.0 mls	1.2 mls
	100	50	1000 µl	8.0 mls	1.0 mls
	150	75	1500 µl	8.0 mls	0.5 mls
	200	100	2000 µl	8.0 mls	0.0 mls

filter sterilize with 0.45 Nalgene

S-9 QUALITY CONTROL

Strain	Mutagen	Mutagen Concentration	Mutagen Revertants/Plate	Mutagen Mean ± S.D.	Spontaneous Revertants/Plate	Spontaneous Mean ± S.D.
TA 98	BP	10 μg/plate				
TA 100	BP	10 μg/plate				
TA 1535	2-AA	3 μg/plate				
TA 1537	BP	10 μg/plate				
TA 1538	BP	10 μg/plate				

Tested By:____________________ Date:____________________

S-9 BIOASSAY

Strain	Mutagen	Mutagen Concentration	S-9 Conc. (μl/plate)	Revertants/Plate	Mean ± Std. Dev.
TA 98	BP	10 μg/plate	0		
			10		
			20		
			30		
			40		
			50		
			75		
			100		
TA 100	DMBA	10 μg/plate	0		
			10		
			20		
			30		
			40		
			50		
			75		
			100		

Tested By:____________________ Date:____________________

APPENDIX Q

Laboratory: Microbiological Associates
5221 River Rd.
Bethesda, MD 20016

Submitter: Andrew M. Losikoff
Marketing Representative
(301)654-3400

Date of submission: December 17, 1980

Omissions: None

Microbiological Associates

5221 RIVER ROAD • BETHESDA, MARYLAND 20016
TELEPHONE: (301) 654-3400

December 17, 1980

Ms. Stephanie Toney
USEPA, HERL, GTD, GBB (MD-68)
Research Triangle Park, NC 27711

Dear Ms. Toney:

Enclosed is a copy of Microbiological Associates' current summary protocol for the Ames Test. I hope this information will help you and Dr. Claxton in your efforts to prepare an Ames Test protocol compilation. I would very much like to receive a final copy of such a document when it is available. My address is:

Microbiological Associates
5221 River Road
Bethesda, Maryland 20016

If you have any further questions, don't hesitate to call me at (301) 654-3400.

Sincerely yours,

Andrew M. Losikoff

Andrew M. Losikoff
Marketing Representative

AL/jkm
Enclosure

A UNIT OF Whittaker CORPORATION

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SUMMARY PROTOCOL 1506.102

SALMONELLA/MAMMALIAN MICROSOMAL ASSAY

1.0 PURPOSE
The purpose of this study is to evaluate the mutagenic potential of the test material based on its ability to induce back mutations at selected loci of several strains of Salmonella typhimurium in the presence and absence of exogenous metabolic activation.

2.0 TEST ARTICLE

2.1 Name (or code):

2.2 CAS Registry Number:

2.3 Description of strength, purity, composition: see attached Chemical Characterization Form.

3.0 SPONSOR

3.1 Name:

3.2 Address:

3.3 Authorized Representative:

4.0 TESTING FACILITY

4.1 Name: Department of Genetic Toxicology
Microbiological Associates

4.2 Address: 5221 River Road
Bethesda, Maryland 20016

4.3 Study Director:

5.0 TEST SYSTEM
Five specially constructed mutants of S. typhimurium (TA1535, TA1537, TA1538, TA98, TA100) were selected for their sensitivity and specificity in being reverted from a histidine requirement back to prototrophy by a wide variety of mutagens (Ames et al., Mutation Research 31: 347-364, 1975) and were received from Dr. B. Ames, Berkeley, California.

6.0 EXPERIMENTAL DESIGN
Five histidine-requiring strains of S. typhimurium will be treated with five concentrations of the test article as well as positive and negative (solvent) controls in the presence and absence of mammalian activating enzymes in soft agar above a minimal growth medium. All test and control doses are to be plated in triplicate. After 46-48 hours, the number of cells which have undergone a mutation to histidine-independence are estimated by enumeration of macroscopic colonies.

Microbiological Associates

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6.1 Dosages

The solubility, toxicity, and dose range of the test article will be determined according to our standard laboratory procedures (1506.99, 1506.918) prior to the mutagenicity assay. Whenever possible, 5 mg/plate or the LD_{50} concentration plus four decreasing doses will be tested.

6.2 S-9

The S-9 is prepared according to our standard laboratory procedure (1506.919) (Ames *et al*., Mutation Research 31: 347-364, 1975). Each preparation of S-9 is assayed for aryl hydrocarbon hydroxylase activity, total protein content and ability to metabolize 2-aminoanthracene to forms mutagenic to all five tester strains of bacteria.

6.3 Controls

6.3.1 Sterility controls

Sterility control plates will be included for each of the five concentrations of test material and for the S-9 mix.

6.3.2 Negative control

The solvent vehicle for the test article will be used as the negative control.

6.3.3 Positive controls

In the assay without exogenous metabolic activation, sodium azide will be used as the positive control for TA1535 and TA100, 9-aminoacridine for TA1537 and 2-nitrofluorene for TA1538 and TA98. For the assay with exogenous metabolic activation, 2-aminoanthracene will be used for all five strains.

7.0 METHODS

The assay is performed by the method of Ames *et al*. (Mutation Research 31: 347-364, 1975) with the addition of several recommendations by DeSerres and Shelby (Science 203: 563-565, 1979) and Mattern and Greim (Mutation Research 53: 369-378, 1978).

7.1 Mutation Assay Without Exogenous Metabolic Activation

Briefly, 0.1 ml of overnight (16-20 hour) broth cultures of *S. typhimurium* are added to 2 ml molten top agar supplemented with 10 ml 0.5 mM histidine - 0.5 mM biotin per 100 ml. The appropriate dose of test chemical, solvent, or control (0.1 ml) is added. The tubes are vortex-mixed and the contents poured onto minimal bottom agar plates. The overlay agar is evenly distributed over the base plate and allowed to harden. All treatments are performed in triplicate. Plates are incubated 46-48 hours at 37±2°C afterwhich revertant colonies are counted.

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7.2 Mutation Assay With Exogenous Metabolic Activation

On the day of the assay, the S-9 is mixed with the cofactor pool to contain: S-9 (100 µl/ml cofactor pool), 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP, and 0.1 M sodium phosphate, pH 7.4. S-9 mix is sterile filtered and stored on ice until used. The mutation assay is performed in the same way as that described in 7.1, except 0.5 ml of the S-9 mix is added to the overlay agar immediately before it is mixed and poured. Plates are incubated 46-48 hours at 37±2°C afterwhich revertant colonies are counted.

7.3 Data Handling

Since a predictable bias exists between manual and automatic colony counting, an adjustment of machine-produced counts will be made to provide a reliable estimate of the actual count. All automatic colony counts between 12 and 1935 will be corrected by the following formula: $y = -7.4 + 1.606x - (7.1 \times 10^{-4})x^2 + (6.31 \times 10^{-7})x^3$, where y = corrected counts and x = machine counts. No correction will be required for machine counts less than 12 and all machine counts greater than 1935 will reported as too numerous to count (TNTC).

8.0 EVALUATION OF TEST RESULTS

The number of revertant colonies from each set of triplicate plates will be averaged and the standard deviation calculated. The average number of revertant colonies found in the test article plates will be compared to those found with the solvent control. A compound will be considered a mutagen if a dose related increase in the number of revertant colonies is found. The first dose level considered for the increase must be three (3) times the solvent control.

9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

9.1 All sterility controls, including the five concentrations of the test article and S-9 mix, must be negative for bacterial growth.

9.2 The average of the controls for spontaneous mutations must be within the following acceptable range: TA1535, 6-40; TA1537, 3-16; TA1538, 6-35; TA100, 80-250; TA98, 10-60.

9.3 The positive control plates (i.e. sodium azide for TA1535 and TA100, 9-aminoacridine for TA1537, and 2-nitrofluorene for TA1538 and TA98 for the assay without exogenous metabolic activation; 2-aminoanthracene for all strains in the presence of exogenous metabolic activation) must have five (5) times the number of colonies as the solvent control.

10.0 ANALYSIS OF TEST ARTICLE
The sponsor will be directly responsible for selection of the method by which determination and documentation of the analytical purity and composition of the test article and the strength of the dosing solutions is to be ascertained.

11.0 RECORD AND SAMPLE ARCHIVES

11.1 Records
Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates.

11.2 Sample
All residual test material will be held in storage for a minimum of two years unless the sponsor requests it to be returned or stability warrants earlier disposal.

SPONSOR APPROVAL ______________________ signature ____________ date

STUDY DIRECTOR ______________________ signature ____________ date

"These protocols are the property of Microbiological Associates(MA) and may not be copied, abstracted, or reproduced in any way or used without prior written consent of Microbiological Associates."

APPENDIX R

Laboratory: Monsanto Research Corporation
1515 Nicholas Rd.
Dayton, OH 45407

Submitter: JoAnne Gridley
Bioanalytical Laboratory
(513)268-3411

Date of submission: December 4, 1980

Omissions: None

Monsanto

MONSANTO RESEARCH CORPORATION
Dayton Laboratory
1515 Nicholas Road
Dayton, Ohio 45407
Phone: (513) 268-3411
TWX 810-459-1681

December 4, 1980

Ms. Stephanie Toney
USEPA, HERL, GTD, GBB (MD-68)
Research Triangle Park, NC 27711

Dear Ms. Toney:

Here are the copies of our current Ames Test Protocol and Standard Operating Procedure as requested. Please send us a copy of the compilation when completed.

Sincerely,

JoAnne Gridley

JoAnne Gridley
Bioanalytical Lab.

a subsidiary of Monsanto Company

MONSANTO RESEARCH CORPORATION
Box 8, Station B
Dayton, Ohio

STANDARD OPERATING PROCEDURE (Document #B-002-R2)

ANALYTICAL PROCEDURE FOR THE AMES/*SALMONELLA* MUTAGENICITY TEST

Prepared by: JoAnne Gridley

Date: October 13 1980

Approved by: William D Ross

Date: October 13, 1980

Quality Assurance Unit Approval: Elaine Santana

Date: October 13, 1980

Lab Director Approval: ______ Issue Date: 10/13/80

Date: October 13 1980 No. of Pages: 18

RECORD OF AMENDMENTS

No.	Date	Type/Description
1	9/19/79	Section 4.8.2, Line 5 ... up to 25 mg will be used.
2	9/19/79	Section 4.8.2, Line 5 ... up to 25 µL.
3	10/13/80	Entire document revised.

Note: MRC's standard operating procedure for *Salmonella* was adapted from Monsanto Company S.O.P. #L-58081-C016.

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1. PURPOSE AND SCOPE

The method to be used at the Bioanalytical Laboratories of Monsanto Research Corporation (MRC) for the determination of mutagenicity according to the Ames/*Salmonella* Test is given in detail in this document.

2. BACKGROUND INFORMATION

2.1 The section following describes the specific steps and requirements of the MRC laboratory procedure. All work also shall be generally conducted according to standard MRC practices for safety, recording of data, and other Good Laboratory Practices contained in relevant documents of this series.

2.2 This procedure will be used to determine mutagenicity of test substance towards *Salmonella* tester strains in the presence of and in the absence of a mammalian microsomal activation system.

2.3 Media and handling procedures are those described by Ames et al. [1].

3. MATERIALS

3.1 Nutrient Broth and Nutrient Agar

8 g/L DIFCO nutrient broth
5 g/L NaCl

The broth and salts are added to water and heated to solution. The solution is aliquotted into 125 mL glass erlenmyer flasks or 250 mL polycarbonate (non-ethylene oxide sterilized) flasks (i.e., 50 mL of solution can be added per flask), and then autoclaved. Nutrient agar plates can also be prepared by adding 15 g/L Difco Bacto agar to the components listed above.

Note: 25 g/L Oxoid broth #2 can be used as an alternative growth medium. After preparation and autoclaving, a filter sterilized stock solution of L-Histidine is added to the cooled broth to a final 30 μg/mL concentration. 15 g/L agar can also be added for oxoid agar plates.

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3.2 Minimal Glucose Medium Plates

Compound	g/L
Citric Acid·H_2O	2.0
$MgSO_4 \cdot 7H_2O$	0.2
K_2HPO_4 (anhydrous)	10.0
$NaNH_4HPO_4 \cdot 4H_2O$	3.5
Difco-Bacto Agar	15.0
Glucose (Dextrose)	20.0

Salts (citric acid, $MgSO_4 \cdot 7H_2O$, K_2HPO_4 and $NaNH_4HPO_4 \cdot 4H_2O$) are prepared separately in 50X concentration and stored at room temperature. A separate solution of agar in distilled water is autoclaved. A separate solution of glucose in distilled water is autoclaved (or filter sterilized) and added to make a final concentration of 20.0 g/L for glucose and 15.0 g/L for agar. 20 mL of freshly autoclaved 50X salts stock is added per liter of the glucose-agar solution. The media (25 to 30 mL) is added to petri dishes (100 x 15 mm plastic petri dishes, non-ethylene oxide sterilized). Plates are dried for 48 hours at room temperature and stored at 4°C until 16-24 hours before use at which time the plates are pre-incubated at 37°C. Plates should be discarded if there is visible contamination or if they are excessively dry.

3.3 Histidine-Biotin Top Agar

Top agar (0.6% w/v Difco-Bacto agar, 0.5% NaCl) is autoclaved and stored in 100 or 200 mL volumes at refrigeration temperature. Before use the top agar is dissolved by heating and 10 mL of a sterile solution of 0.5 mM L-histidine·HCl, 0.5 mM biotin is added per 100 mL of top agar. Histidine-biotin solution is sterilized by filtration through a 0.2 µm filter and stored in a refrigerator. 25 mL of sterile 0.2 M phosphate buffer or 0.85% saline is added per 100 mL top agar used for assay without S-9 activation (to replace the volume addition of 0.5 mL S-9 per 2 mL top agar in assays with activation). 2.0 mL quantities of top agar with histidine and biotin can be pre-aliquotted into sterile 13 x 100 mm tubes and refrigerated prior to testing. Just before use, the agar can be heated in the tubes. 0.5 mL of buffer can then be added per tube of top agar that is being prepared for assay without microsomal activation.

When large volumes of complex environmental liquids are tested (i.e., 1 mL per plate), the top agar can be prepared at 2X concentration and 1.0 mL of top agar can be diluted back with 1.0 mL of the sample or samples' dilution. Also, if viable counts of bacterial cultures are necessary, top agar can be supplemented with higher concentrations of histidine (60 µg/mL).

3.4 Minimal Glucose Plates with Limiting Histidine and Biotin

Two mL of a 0.05 mM histidine-biotin top agar poured onto a minimal glucose plate is referred to as a minimal plate with limiting histidine and non-limiting biotin. Other components may also be present in the assay (S-9 mix, buffer, positive and negative standards, samples, solvents).

3.5 Rat and Mouse Liver Microsomal Enzymes

Rat and mouse liver S-9 preparations are purchased commercially from Litton Bionetics, Kensington, MD. These preparations are from the livers of Aroclor 1254-induced male Sprague-Dawley rats and Aroclor 1254-induced male CD-1 mice. Each lot of enzymes is defined with a lot number, the date prepared, the protein content, benzo(a)pyrene hydroxylase activity, and the dose response of two appropriate positive (mutagenic) standards using at least five S-9 concentrations and using at least two tester strains (TA98 and TA100).

4. PROCEDURE

4.1 Growth of Cultures

All cultures used for testing should be inoculated from a few single colonies on a minimal agar plate supplemented with biotin (0.1 mL of a 0.5 mM d-biotin solution) and histidine (0.1 mL of a 0.1 M L-histidine solution) or a nutrient agar plate. Such isolated colonies (master plates) should be prepared by streaking and cross-streaking bacteria obtained from a permanent culture (soft agar or frozen permanent) onto minimal agar plates supplemented with biotin and histidine or nutrient agar plates. Single colonies may then be used to inoculate nutrient broth cultures. Since R factors tend to be lost on storage, 0.1 mL of an 8 mg/mL ampicillin (in 0.02N NaOH) solution is added per plate used to grow and maintain strains TA98 and TA100. Nutrient broth cultures should be grown overnight (maximum 18 hours) with shaking at 37°C. Nutrient broth cultures may be stored at 4°C, and used for testing for up to one week, but daily cultures are prepared unless otherwise noted. Before using a bacterial culture for testing, a Petroff-Hauser cell count or an optical density (using a Bausch and Lomb

Spectronic 20) is taken to approximate cell density. Preferred cell density should be in the range of 5×10^8 to 5×10^9 cells per mL.

4.2 Storage of Strains

All test strains should be stored at -70°C to -80°C, as frozen permanents. Frozen permanents are prepared from nutrient broth cultures which have been checked for appropriate phenotypic properties. About 8.0 mL of nutrient broth culture and 0.7 mL of dimethylsulfoxide (or 0.08 mL of DMSO per mL of culture) are frozen on dry ice and acetone in sterile, glass, screw-capped test tubes to make a frozen permanent. It is recommended that at least three frozen permanents be made for each test strain. One permanent is used as a master copy and the others are used for inoculating cultures used to prepare master plates (single isolated colonies for daily inoculation).

4.3 Pouring Plates

Molten histidine-biotin top agar is dispensed, while hot, in two mL portions into sterile test tubes in a bath at 45°C (or pre-aliquotted 2 mL volumes in tubes are used). Tubes to which no S-9 mix will be added have 0.5 mL of 0.2 M phosphate buffer or 0.85% saline added to make up the volume difference. After allowing sufficient time for the histidine-biotin top agar to come to 45°C, test sample or test solvent or positive standard (if appropriate), bacteria (0.1 mL of an overnight nutrient broth culture), and S-9 mix (if appropriate) are added in that order. Contents are rapidly mixed and poured onto minimal glucose plates. Plates are allowed to incubate at 37°C for at least 48 hours before observations are made.

4.4 Verification of Test Strains

Strains used in the test will include TA1535, TA1537, TA98, and TA100. Strain TA1538 can be added for use when specified. Procedures for culture and verification of strain integrity are given below.

4.4.1 Crystal Violet Sensitivity

A sterile filter paper disc containing crystal violet (10 µL of a 1 mg/mL solution) is placed on a petri plate of Ames bottom agar containing 0.1 mL of the bacterial culture to be tested in a thin top agar overlay supplemented with 60 µg/mL L-histidine and 0.05 mM biotin.

After incubation at 37°C for 24-48 hours, the absence or presence of a zone of growth inhibition surrounding the disc is noted and recorded. Growth of strains containing the deep rough (rfa) mutation are inhibited by crystal violet.

4.4.2 Ampicillin Sensitivity

A disc containing ampicillin (BBL-10 mcg) is placed on a petri plate of minimal agar containing 0.1 mL of the bacterial culture to be tested in a thin top agar overlay supplemented with 60 µg/mL L-histidine and 0.05 mM biotin. After incubation at 37°C for 24-48 hours, the absence or presence of a zone of growth inhibition surrounding the disc is noted and recorded. Growth of strains containing the R factor is not inhibited by ampicillin. (For a more quantitative assessment of ampicillin sensitivity, 0.1 mL of 10^{-5} or 10^{-6} dilutions of bacterial cultures can be plated with top agar supplemented with 60 µg/mL L-histidine and 0.05 mM biotin onto minimal agar plates containing 25 µg/mL ampicillin. Results are compared to viability counts determined from the cultures by plating the same dilutions under the same conditions with the exception that ampicillin is not incorporated into the bottom agar).

4.4.3 Histidine Requirement

0.1 mL of 0.1 M L-Histidine solution is spread onto the surface of an Ames (minimal) bottom agar plate. 0.1 mL of 0.5 mM d-biotin solution is spread onto the surface of an Ames bottom agar plate. A third plate is spread with both 0.1 mL biotin and 0.1 mL histidine solutions. A fourth plate is left as is (minimal plating agar). Each strain being tested is streaked onto the four types of prepared agars and incubated for 48 hours at 37°C. Growth on the minimal plating agar and supplemented plating agars is recorded in each case.

4.4.4 UV Sensitivity

Each strain is cross-streaked onto a nutrient agar plate. Each half-plate is covered with a cardboard card or sheet of heavy paper and UV irradiated for 6 seconds (8 seconds for strains containing the R-factor) at a distance of 65 to 70 cm from a G.E. Sterilamp G36T6L source.

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Plates are incubated at 37°C for 12-24 hours. Growth or inhibition of growth on the half of the plate that was UV treated is noted and recorded. Inhibition of growth by UV treatment indicates the presence of the uvrB deletion.

4.4.5 Spontaneous Reversion Rate

Spontaneous reversion rate is checked by plating in triplicate - 0.1 mL of each culture being tested with 2 mL of top agar overlay containing biotin (0.05 mM) and a trace of histidine (0.05 mM) onto Ames basic (minimal) plating agar. Plates are incubated at 37°C for 48 hours. The number of spontaneous revertants are recorded.

4.5 Preparation of S-9 and S-9 Mix

Liver microsomal fractions (S-9) will be prepared from rats or mice induced with Aroclor 1254, unless otherwise specified. Preparation of S-9 fractions and S-9 mixes will be as described by Ames et al. [1]. The amount of S-9 fraction (0.04 to 0.1 mL per mL of S-9 mix) will be determined for each lot of S-9 by maximizing response to positive control mutagens requiring activation in plate incorporation assays. Commercial S-9 preparations prepared by the above techniques are acceptable. The following details are given for preparation of S-9 and S-9 mix.

4.5.1 Male rats (Sprague-Dawley) weighing about 200 g are injected i.p. with Aroclor 1254 (200 mg/mL in corn oil) at a dosage of 500 mg/kg five days before sacrifice. Food (Purina Lab Chow) is removed 12 hours before sacrifice. Rats are sacrificed and liver S-9 is prepared. Because individual variations in animals may affect activation capability, at least three rat livers should be pooled in one S-9 preparation. Mouse strains which are inducible for aromatic hydrocarbon hydroxylase or dimethylnitrosamine-activating activity should be used. Such strains include the following: C57BL/6N, C58/J, C57BL/6J, CD-1, C57L/J, C3H/HeN, OF-1. Other strains may be used provided that it is shown that increased activity is produced upon Aroclor 1254 treatment and that the S-9 preparations have good capability towards such compounds as dimethylnitrosamine, and possibly chlorinated organic compounds.

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4.5.2 Microsome preparations should be conducted in the following manner using sterile techniques and instruments. Livers should be placed in beakers containing approximately 1 mL/g wet weight 0.5 m KCl, weighed and homogenized in 3 mL/g wet weight 0.15 m KCl. The homogenate is centrifuged for 10 minutes at 9,000 x g in a refrigerated centrifuge. The supernatant solution (S-9 fraction) is decanted and stored in 2 mL or 5 mL portions at -70°C to -80°C after being quickly frozen on dry ice. Each vial can be used only once. Vials should not be thawed and refrozen. Prior to use in testing, the S-9 fractions should be characterized for their ability to activate standard compounds to mutagenic forms. An appropriate procedure is to first set a constant amount of standard compound with different levels of S-9. A variety of standard compounds should be used (e.g., benzo(a)pyrene, 2-aminoanthracene and acetylaminofluorene or Aflatoxin B1). At least two tester strains should be used under these conditions (TA98 and TA100). S-9 dose response curves should be used in establishing the quality of the S-9 preparation.

4.5.3 S-9 Mix

S-9 Mix contains the following per mL: S-9 (0.04 to 0.1 mL), 8 μmoles $MgCl_2$, 33 μmoles KCl, 5 μmoles glucose-6-phosphate, 4 μmoles NADP and 100 μmoles sodium phosphate, pH 7.4. Stock solutions of NADP (0.1 M) and glucose-6-phosphate (1M) are prepared with sterile water, filter sterilized, and stored frozen at -70°C to -80°C. The salt solution (0.4 M $MgCl_2$, 1.65 M KCl) and phosphate buffer (0.2 M, pH 7.4) are autoclaved and stored refrigerated. S-9 mix is prepared fresh each day and may be kept several hours on ice before use. If S-9 mix is found not to be sterile, it may be sterilized by filtration, provided that positive controls are not seriously affected.

4.6 Preparation of Positive Standards (Mutagens)

Solutions of standard compounds (mutagens) are prepared by diluting a freshly prepared stock solution in sterile septum-crimped vials. Appropriate positive standards are prepared every 6-8 weeks (sooner if necessary) and stored at room temperature in aluminum foil-covered vials. Standards are logged into a positive standards log book. A specific QA/QC sheet of standards preparation information is also filed. Appropriate positive standards are tested concurrently with all *Salmonella* assays both in the presence and absence of microsomal activation. Results generated with all standards are routinely recorded and monitored.

4.7 Preparation of Sample

Testing will not begin on a sample until the test sample is properly identified and labeled. Whenever possible, sample identity of pure compounds will include sample composition, sample purity, sample stability, and any known chemical, physical, or toxic properties exhibited by the sample. Samples will be properly logged into a sample log-in system and unique log and study numbers will define the sample as well as the type of test to be performed with the sample. Samples will be properly labeled and stored in a vented refrigerator unless otherwise specified. Prior to testing, the sample will be checked for microbial sterility by plating an aliquot of the sample onto a complex medium and incubating for 24-48 hours at 37°C. Sample solubility will be tested in the following solvents (in order of preference): water, DMSO, ethanol, acetone, or the di-methyl ethylene glycols.

Samples are prepared fresh on the day of testing by making specified dilutions of a stock of sample made in the appropriate solvent. The dilutions and stock are prepared in sterile septum crimped vials or other appropriate containers and the information concerning each dilution is properly recorded. Equal volumes of sample stocks or dilutions are added to each plate in the testing scheme by removing aliquots from the crimped vials with an appropriate syringe.

4.8 Spot Test

Prior to plate incorporation tests, a spot test will be conducted on all samples tested unless otherwise specified. The spot test will use the four test strains TA98, TA100, TA1535 and TA1537 (strain TA1538 can be added when specified). Strains will be tested in the absence of microsomal preparations and in the presence of S-9 mixes prepared from rat liver S-9 fraction (induced with Aroclor 1254) and mouse liver S-9 fraction (induced with Aroclor 1254). Each strain and microsome condition will be tested with one plate. Appropriate positive and negative controls will be run. Details of the spot test are given below.

4.8.1 The bacterial strains TA98, TA100, TA1537 and TA1535 will be used (unless otherwise specified). Each strain will be used in the absence of S-9 mix and in the presence of rat liver S-9 mix and mouse liver S-9 mix. Bacterial culture (0.1 mL of an overnight nutrient broth culture) and 0.5 mL of S-9 mix (if appropriate) will be added to 2 mL of 0.05 mM histidine-biotin top agar and poured on minimal glucose plates. The top agar is allowed to set (about 10 minutes).

4.8.2 Compounds will be placed in the center of the plate. Liquids will be placed directly in the center of the plates on sterile paper discs. Solids will be dissolved in water, or DMSO, or an appropriate solvent. A maximum total of 0.1 mL of solution will be used (containing a maximum amount of compound, up to 25 mg for pure solids). Up to 25 µL of pure liquid samples should be used. Higher quantities of dilute or complex test samples can be added when specified. If the amount of sample used does not permit any bacterial growth on the plate, the amount will be reduced until some background lawn growth is observed. After 48-hour incubation at 37°C, the number of colonies on the plate will be counted. Any apparent effects on or zones of inhibition of background growth will be noted and measured. A positive mutagenic response will be a halo of revertant colonies surrounding the sample/disc at a distance from the sample gradient optimum for the mutagenic response. In case volatile compounds are used, the plates will be placed in sealed containers to prevent contamination of other plates in the incubator.

Note: A positive response of a volatile compound may produce an increase in revertants per plate without the halo effect.

4.9 Toxicity Test

A toxicity screening test will be performed on all samples prior to plate incorporation assays unless otherwise specified. The toxicity test will first be performed at a level of 10, 3, 1, 0.2, 0.04, and 0.01 mg for pure solids (or µL for pure liquids) per plate using TA100 with and without microsomal activation. (Dilute environmental water effluents can be tested at 1, 0.3, 0.1, 0.02, 0.004 and 0.001 mL per plate or other specified concentrations). Minimal glucose plates with 0.05 mM histidine-biotin top agar will be used. Appropriate negative and solvent controls will be run concurrently. Positive controls can be run also but are not required. A toxic response is indicated when the background lawn is non-existent or reduced to discrete individual "pinpoint" colonies. Decrease in background growth will also be noted. If a significant toxic response is observed at the lowest concentration, lower concentrations will be tested for toxicity. If mouse microsomal enzymes are either specified or indicated by the spot test the toxicity test will be performed with mouse microsomal preparations prior to plate incorporation testing.

4.10 Plate Incorporation Tests

Plate incorporation tests shall be conducted on all compounds tested unless otherwise specified. The results of the toxicity test or solubility range will be used to specify the concentrations tested. If no toxic response is indicated at 10 mg/plate then 10 mg/plate will be the maximum concentration tested (10 µL in the case of liquids). Higher concentrations may be tested for more dilute or complex environmental samples such as water effluents. If a toxic response is indicated, then the maximum concentration tested will be the lowest tested concentration giving a significant toxic response (moderate level of toxicity such as pinpoint background lawns) or one-half the lowest concentration giving severe toxicity (such as absence of lawns or severely limited background lawns). If a compound is not soluble at levels less than 10 mg/plate (or the highest concentration assessed in the toxicity assay) then the maximum concentration tested will be the lowest concentration at which the compound is insoluble as determined by a series of concentrations similar to those used for toxicity testing. (If the sample is insoluble at extremely low concentrations, the study director may authorize testing the sample in the insoluble range if the sample precipitant does not interfere with the ability to count revertant colonies).

4.10.1 The concentrations to be tested will be C, 0.3C, 0.1C, 0.02C, 0.004C and 0.001C, where C is the maximum concentration. C may be different in the presence and absence of microsomal preparations.

4.10.2 All compounds will be tested in the presence and absence of microsomal enzymes unless otherwise specified. A microsomal preparation from liver of rats induced with Aroclor 1254 will be used unless otherwise indicated. A microsomal preparation from livers of mice induced with Aroclor 1254 wll be used in the place of rat microsomal preparations if this is specified or if results from the spot test indicate that mouse microsomal preparations are uniquely or preferentially capable of metabolizing the compound to a mutagenic form.

4.10.3 Three replicate plates will be prepared for each concentration. A minimum of three replicate plates will also be prepared for each solvent control. At least one plate each will be used for non-solvent control and for positive controls.

4.10.4 If a positive response or responses are indicated in the spot test then the strain which exhibits the response or strain which exhibits the greatest response will be tested first in the plate incorporation assay. The test will be conducted with the stated concentrations, both with and without microsomes unless otherwise specified. If a positive mutagenic response is indicated (as defined below), no further tests will be done unless specified.

4.10.5 If no positive response is indicated in the spot test, the compound will be tested in the following manner. Compounds will be tested using TA98, TA100, TA1535, and TA1537 in the presence and absence of microsomal preparations. Strain TA1538 may also be tested when specified. If a positive response (as defined below) is obtained with any of these strains, no further testing will be performed unless specified.

4.10.6 A retest will be performed under certain conditions determined in the initial test. Specifically, only one or two concentrations may give a response which is significant at the $p \leq 0.01$ level, or three concentrations may give a significant response but no dose response is observed. In these cases, retesting will be required before a judgment is made as to whether or not a positive response has been obtained. The retest shall be conducted with all strains which exhibit a significant ($p \leq 0.01$) response for at least one treatment concentration. The retest shall consist of using the concentrations of 0.5x, x and 1.3x where x is the concentration initially observed as giving the most apparently significant response. If x happens to be the maximum concentration permitted by toxicity or solubility then 0.5x, 0.7x and x will be used. Each concentration should be tested with three replicates. The retest should be conducted either with and/or without microsomal preparations, depending on which condition gave the apparently significant response in the initial test. Solvent controls should be run at least in triplicate. At the minimum, single plates will be used for non-solvent and positive controls.

4.10.7 Details of the plate incorporation test are given below:

4.10.7.1 Samples will be dissolved in sterile water, if possible. If water is not a suitable solvent, DMSO (dimethyl sulfoxide) will be used. If neither DMSO or water can be used, ethanol, acetone, or the dimethyl ethylene glycols, E-141 or E-181, can be used provided that less than 100 µL is used per plate (only 50 µL/plate the maximum with acetone). Dilutions of the compound should be prepared such that the same volume can be used for each concentration tested.

4.10.7.2 The plates should be poured by adding (in order) test sample, 0.1 mL bacterial suspension (overnight nutrient broth culture), and 0.5 mL S-9 mix (if appropriate) to 2 mL of 0.05 mM histidine-biotin supplemented top agar at 45°C. Two mL top agar should also be supplemented with 0.5 mL of 0.2 M phosphate buffer or 0.85% saline when S-9 is not being added. Top agar samples should be mixed rapidly and poured onto minimal glucose plates. Plates are heat-sealed into Kapak bags (with one strain-microsome combination in one bag). Colonies should be counted after 48 hours incubation (minimum) at 37°C. Colonies can be counted with an automatic colony counter provided that calibration of the counter is established and checked periodically.

4.10.7.3 Positive, negative and solvent controls shall be run concurrently with the test plates. At the minimum, for solvent controls, three replicate plates shall be used for each strain both with and without S-9 mix and with the same amount of solvent used as in the test. At least one additional plate without solvent should be used as a negative control per strain/microsome combination. All controls should be prepared on the same day as the test compounds, using the same bacterial cultures and the same S-9 mix. Suggested limits for spontaneous revertants on solvent and negative control plates are:

Strain	+ S-9 Mix	- S-9 Mix
TA1535	12 ± 10	15 ± 10
TA1537	12 ± 10	10 ± 8
TA98	30 ± 15	25 ± 15
TA100	150 ± 75	150 ± 75

Solvent control results are monitored routinely and are used to statistically establish an acceptable response range for each strain/microsome combination used in the laboratory. If solvent control results fall outside of the established range, then a retest using the appropriate strain/microsome condition is required.

4.10.7.4 Positive controls will be run on single plates at the minimum. The following compounds are appropriate for use as positive controls:

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Strain	S-9 Mix	Amount and Compound
TA98	-	2 µg 2-nitrofluorene*
TA100	-	100 µg methyl methane sulfonate
	-	2 µg 2-nitrofluorene *
TA1535	-	20 µg N-hexyl-N-nitro-N-nitrosoguanidine
	-	9 mg $NaNO_2$*
TA1537	-	50-150 µg 9-aminoacridine*
TA98	+	5 µg benzo(a)pyrene*
	+	1 µg 2-aminoanthracene
TA100	+	5 µg benzo(a)pyrene*
	+	1 µg 2-aminoanthracene
TA1535	+	5-20 µg 2-aminoanthracene*
TA1537	+	5-20 µg 2-aminoanthracene*

4.10.7.5 The compounds marked with an asterisk (*) are suggested compounds and are presently used as positive controls at MRC. Special handling precautions should be taken with all of the compounds since all are mutagenic and suspect carcinogens. The exact concentration of positive mutagen used to elicit a response is documented with each use. The positive responses are monitored routinely and used to establish an acceptable range of response for each strain/microsome combination within this laboratory. If a standard mutagen fails to elicit a response within the established range, the positive control is considered unacceptable and a retest is required.

4.10.8 Statistical Analysis

4.10.8.1 All replicate treatment data (revertants-per-plate) will be transformed using $\log_{10}$ to reduce scatter and produce a more homogeneous variance among treatment types. Bartlett's test will be used to verify homogeniety of variance. A one-tailed t-test will be used to test the hypotheses:

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1. H_o: $\overline{X}_T = \overline{X}_C$ (treatments same as controls)

2. H_1: $\overline{X}_T > \overline{X}_C$ (treatments greater than controls)

3. $\rho = 0.01$ (significance level of test)

4. Critical region
   $T > t_\rho$ ($T_{calculated} > T_{table}$)

All tests will be done at the high level of significance (p = 0.01). Therefore, if we can reject the null hypothesis (H_o) and conclude that the response of the treatment is significantly greater than the response of the control, the probability of committing a Type I error is 0.01. In other words, the probability of rejecting the null hypothesis when it is true is only 0.01. (Note: the actual probabilities will also be reported). Any sample treatment results that are significantly greater than solvent control results (p = 0.01) are designated so on a statistical print out of each test.

4.10.8.2 Dose response will be evaluated as follows:

Dose response will be evaluated for all strain/microsome conditions in which at least one treatment is significantly higher than controls (p = 0.01). Dose response will not be evaluated with two or fewer treatments. Dose response will be analyzed using the linear regression of log concentration vs log revertants per plate. A t-test will be used to evaluate significance of dose-response. Dose response is considered significant if p = 0.01. A lack of fit-test will be performed and, if lack of fit is significant (p = 0.01) dose response analysis will be repeated omitting treatments significantly smaller than control. Grubb's analysis for outliers will also be performed. Any outliers detected will be omitted and data reanalyzed for dose-response.

4.10.9 Evaluation of Test Results

The following criteria will be used to determine whether a positive response has been obtained:

4.10.9.1 A positive outcome on initial test consists of three or more treatments on at least one test strain, with or without microsomes, significantly greater than the control ($p = 0.01$) and the dose-response also significant ($p = 0.01$).

4.10.9.2 A negative outcome on initial test occurs when none of the treatments on any of the four test strains with or without microsomes is significantly greater than the control ($p = 0.01$). In this event, the outcome of the dose-response is irrelevant.

4.10.9.3 A retest is indicated if on initial test, for at least one test strain, one or two treatments are significantly greater than the control ($p = 0.01$); if three or more treatments are significantly greater than the control ($p = 0.01$), but the dose-response is not significant; if fewer than two treatments are available for the dose-response test, or if all quality control parameters do not meet designated (GLP) guidelines.

4.10.9.4 A positive outcome on retest occurs when the cumulative numer of treatments (on initial test and retest) significantly greater than the control ($p = 0.01$) is three or more and the dose-response relationship is significant ($p = 0.01$) for initial test or retest or both. Data from initial test and retest will be analyzed separately.

4.10.9.5 A negative outcome on retest is indicated if the cumulative number of treatments significantly greater than the control ($p = 0.01$) is less than three or if the dose-response is not significant ($p = 0.01$) for either initial test or retest.

4.10.9.6 A second retest is required if and only if in the first retest, fewer than two treatments are available for the dose-response analysis or if all quality control parameters do not meet GLP guidelines in the retest.

4.10.9.7 If a second retest is required, the results from the first retest will be ignored.

5. REFERENCES

1. Ames et al., Mutation Research 31, 347-364 (1975).

STUDY PROTOCOL
FOR
AMES/*SALMONELLA* MUTAGENICITY ASSAY

BIOANALYTICAL LABORATORY
MONSANTO RESEARCH CORPORATION
Box 8, Station B
Dayton, OH 45407

For Test Compound: ______________________________

Sponsor Project No.: ______________________________

Sponsor: ______________________________

Address: ______________________________

Proposed Starting Date: ______________________________

Proposed Completion Date: ______________________________

Proposed Final Report Submitted: ______________________________

Proposed Dates will be Honored if Test Samples are Submitted to MRC by: ______________________________

Protocol Submitted By: ______________________________

Study Director: ______________________________

Date: ______________________________

Protocol Approved By: ______________________________

Date: ______________________________

MRC Study Number: ______________________________

Protocol adapted and condensed from Monsanto Research Corporation's Standard Operating Procedure for Ames/*Salmonella* Mutagenicity Assay, Document #B-002-R2.

October 1980

DMEH6/JOB F

1. PURPOSE

The Ames/*Salmonella* test detects mutagenic activity (gene mutations) for a number of chemicals of a variety of chemical classes, including those requiring mammalian metabolic activation. It is used as a screening system for potential mutagenic and carcinogenic activity in mammals.

2. TEST MATERIALS

2.1 Test Sample:

2.1.1 Test Material Name:

2.1.2 Test Material Log Number:

2.1.3 Test Material Study Number:

2.1.4 The test will not be initiated until the following is established:

2.1.4.1 A unique study number and log number is assigned per sample, and the sample is labeled and stored properly.

2.1.4.2 Sample solubility, stability, and purity information is verified or tested. (For complex environmental samples such as water effluents, determintion of purity, stability and compostion is most likely unfeasible). Checks for gross microbial contamination will be performed.

2.2 Positive Controls

Chemical Name	Strain	With or without S-9 activation
benzo(a)pyrene	TA98 and TA100	With
2-aminoanthracene	TA1535 and TA1537	With
2-nitrofluorene	TA98 and TA100	Without
sodium nitrite	TA1535	Without
9-aminoacridine	TA1537	Without

Positive controls are run concurrently with each assay performed. The dose of each compound required to elicit a mutagenic response is documented and monitored. The numbers of induced revertants per plate are documented and monitored, and statistical ranges of acceptable values for induced revertants per plate are calculated using this laboratory's history of results.

2.3 Test Solvent

Test materials are dissolved in distilled water, dimethyl sulfoxide, ethyl alcohol, anhydrous acetone, or the dimethyl ethylene glycols (E-141 or E-181). Either distilled water or DMSO are the solvents of choice dependent on the solubility of the sample in each. Samples are prepared for testing by making a stock solution of the sample in the appropriate solvent. Serial dilutions of the stock are prepared with the solvent in order to allow equal volumes of dilutions to be added per plate for each concentration tested. (If a complex environmental water sample is being tested, higher concentrations of the neat sample may be added per plate and dilutions may not be necessary).

2.4 Test System

2.4.1 *Salmonella typhimurium* test strains TA98, TA100, TA1535 and TA1537 are used.

2.4.2 Strain genotypes of all cultures used to prepare master plates for daily inoculations or stock cultures that are to be frozen down for maintenance are verified by phenotypic determination of histidine and biotin auxotrophy, crystal violet sensitivity, UV sensitivity, ampicillin sensitivity and spontaneous reversion frequency.

3. PROCEDURES

3.1 Experimental Design

A complete description of the experimental procedures is given in the MRC Standard Operating Procedures for Ames/*Salmonella* Plate Incorporation Test (Document #B-002-R2). The following elements are included:

3.1.1 A toxicity test to determine the maximum concentration permitted (if less than 10 mg or 10 μL per plate in the case of single test compounds or if less than 1 mL per plate in the case of dilute environmental water effluents).

3.1.2 A spot test for determination of activity of volatile compounds and compounds with strong activity in the presence of mouse microsomal enzymes. The test is conducted with TA98, TA100, TA1535 and TA1537 in the absence of an activation system and in the presence of Aroclor-1254 induced rat and mouse liver activation systems.

3.1.3 A plate incorporation test using strains TA98, TA100, TA1535 and TA1537 in the absence and presence of an Aroclor-1254 induced mouse or rat liver activation system.

3.1.4 Concurrent solvent controls are used in all experiments. If water is not used as a test solvent, a water control is also run as a negative control. Positive controls are used in all spot and plate incorporation assays.

3.2 Route of Administration

Test materials are placed in the center of the petri plate (spot test) and incorporated into the top agar (plate incorporation assay and toxicity assay). The former method detects activity for certain volatile compounds and the latter is the most sensitive for many materials.

3.3 Dose Levels

3.3.1 A maximum of 25 mg (or 25 µL) of pure or single test compounds is used per plate in the spot test. (For complex environmental compounds or water effluents, higher concentrations may be tested at the discretion of the study director.) Lower amounts are used if toxicity is excessive.

3.3.2 Whichever is least of the following is the maximum amount per plate tested in the plate incorporation test of pure or single test compounds:

(a) 10 mg or 10 µL
(b) The lowest significantly toxic concentration observed.
(c) The lowest amount observed to be insoluble.

(In the case of dilute environmental water effluents, up to 1.0 mL of sample can be tested per plate if the sample is not found to be toxic at this concentration.) Other concentrations tested are 0.3C, 0.1C, 0.02C, 0.004C and 0.001C where C is the maximum concentration tested.

3.4 Frequency of Tests and Measurements

3.4.1 One replicate is used for each treatment in the toxicity test. Strain TA100 is used with and without a rat liver activation system. Plates are examined visually for toxicity after 48 hours (minimum) at 37°C by examining microscopic background lawns of cell growth. Any sample precipitation or sample interference is also noted. Concurrent solvent controls (in triplicate) and non-solvent controls (one replicate) are used in each assay.

3.4.2 One replicate is used for each treatment in the spot test. Any zone of growth inhibition or effect on background lawns is measured for each plate after 48 hours (minimum) at 37°C. The number of revertant colonies or ring of revertant growth (if present) is also measured. Concurrent solvent controls (in triplicate) and non-solvent controls (one replicate) are used in each assay. Positive controls (one replicate the minimum) are also tested.

3.4.3 Triplicate plates (the minimum) are used for all treatments and the solvent controls in the plate incorporation test. At least one replicate is used for the non-solvent and positive controls. Revertant colonies per plate and toxicity (if any) are measured after 48 hours at 37°C. Any sample precipitation or interference is reported if it should occur.

4. RECORDS

4.1 For all tests performed the following information is recorded (or referenced).

4.1.1 Strain culture identification.

4.1.2 Information on test compound solutions and positive control solutions:

- 4.1.2.1 Preparation date
- 4.1.2.2 Solvent and solvent lot number
- 4.1.2.3 Lot number of chemical standards
- 4.1.2.4 Initials of person preparing solutions
- 4.1.2.5 Dilution volumes
- 4.1.2.6 Final concentration of stocks and dilutions
- 4.1.2.7 Assigned log number of positive standards used

4.1.3 Amount of test material per plate (or spot).

4.1.4 The lot number and concentration of S-9 in the S-9 mix.

4.1.5 Reagent and media log numbers

4.1.6 Sample identification

4.1.7 Date of testing and date of counting

4.1.8 Initials of person performing test and initials of person counting test

4.2 For the toxicity test, the following test results are recorded:

4.2.1 Effects on background lawn growth

4.2.2 Revertants per plate

4.2.3 Sample precipitation or interference (if any)

4.3 For the spot test, the following test results are recorded:

4.3.1 Effects on background lawn growth (if any)

4.3.2 Size of growth inhibition zone (if any)

4.3.3 Qualitative indication of no mutagenic response or revertants per plate. Any rings or clusters of revertant colony growth are noted.

4.4 For the plate incorporation test the following results are recorded:

4.4.1 Toxicity (or any apparent effect on background lawn growth) if present

4.4.2 Insolubility of test material (if observed)

4.4.3 Revertants per plate

5. STATISTICAL METHODS, AVOIDANCE OF BIAS, AND CRITERIA FOR POSITIVE RESPONSE

5.1 Statistical methods are employed to make decisions on test results from plate incorporation tests. These procedures are objective and eliminate bias of subjective judgment of test results. The specific methods used are:

5.1.1 Values of revertants/plate are transformed using log (base 10) for further analysis.

5.1.2 Bartlett's test is performed to determine if significant difference exists among treatment variables.

5.1.3 Treatments are compared with solvent controls using a one-sided t-test and within-levels pooled variance.

5.1.4 Dose response is evaluated for all treatments which are significantly higher (p=0.01) than the solvent controls.

5.2 A positive response in the plate incorporation test is indicated if three or more treatments on the initial test (and/or retest, if performed) are significantly greater than solvent control (p=0.01) and if there is a significant positive dose-response (p=0.01) for the initial test (and/or retest, if performed).

APPENDIX S

Laboratory: National Enforcement Investigations Center
U.S. Environmental Protection Agency
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Denver, CO 80225

Submitter: Dr. John E. Preston

Date of submission: November 26, 1980

Omissions: None

ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF ENFORCEMENT
NATIONAL ENFORCEMENT INVESTIGATIONS CENTER
BUILDING 53, BOX 25227, DENVER FEDERAL CENTER
DENVER, COLORADO 80225

TO See Below

DATE November 26, 1980

FROM Thomas P. Gallagher
Director, NEIC

SUBJECT Transmittal of Method Manual, "Microbial Bioassay for Toxic and Hazardous Materials"

Enclosed for your use is a copy of the subject manual. This manual describes the procedures used by the NEIC to qualitatively screen complex wastes for the presence of mutagenic and potentially carcinogenic chemicals.

These procedures utilize the standard Ames Mutagenicity Assay, however, minor modifications have been made so that the test can be applied to complex waste materials. Work is presently underway to refine and standardize these procedures. In the interim, EPA Regional Laboratories and other program offices with responsibilities for monitoring toxic and hazardous materials, may wish to use these procedures in order to provide a uniform approach in evaluating environmental samples or other complex mixtures.

Should you desire additional copies of this document, a limited number are available at the NEIC. The document is also available from the NTIS (National Technical Information Service, 5285 Port Royal Road, Springfield, Virginia 22161).

Attachment

Addressees:

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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF ENFORCEMENT

EPA-330/9-80-002

MICROBIAL BIOASSAY FOR TOXIC AND HAZARDOUS MATERIALS (AMES TEST FOR MUTAGENICITY)

August 1980

NATIONAL ENFORCEMENT INVESTIGATIONS CENTER
Denver, Colorado

EPA

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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF ENFORCEMENT

EPA-330/9-80-002

NEIC
MICROBIAL BIOASSAY FOR TOXIC AND HAZARDOUS MATERIALS
(AMES TEST FOR MUTAGENICITY)

September 1980

William J. Stang

NATIONAL ENFORCEMENT INVESTIGATIONS CENTER
Denver, Colorado

FOREWORD

Environmental measurements are required to determine the quality of ambient waters and the character of waste effluents. Determination of the toxicity of hazardous wastes has a high priority in the EPA water pollution and hazardous waste control programs. This procedure is a modification of the Ames test; it fills an urgent current need for a rapid screening method for determining mutagenicity and potential carcinogenicity. Federal agencies, states, municipalities, universities, private laboratories and industry should find this manual of assistance in monitoring toxicity in the environment.

Thomas P. Gallagher
Director, NEIC

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PREFACE

This manual on the Ames Mutagenicity Test was developed for use by the NEIC. The document is provided as a uniform laboratory developmental method for rapid screening of complex environmental mixtures for mutagens and potential carcinogens. Publication of this procedure acknowledges the potential of the Ames test to determine some of the toxic effects of waste materials.

Publication also culminates a series of historical events that make use of the bioassay possible and refinement imperative:

- The use of epidemiological studies, long-term bioassays, and short-term in vitro tests, such as the Ames test, were first authorized in the Federal Water Pollution Control Act, Amendments of 1972 and 1977. The Drinking Water Act of 1974, and the Toxic Substances Control Act of 1976 to determine mutagenic effects of toxic and hazardous materials.

  The definition of toxic pollutants in the Clean Water Act of 1977 includes substances which cause mutations and cancer.

- In 1979 the Interagency Regulatory Liaison Group and the Regulatory Council encouraged the development of short-term screening tests using microorganisims to detect carcinogenisis.

- In 1978 and 1979, the EPA Biological Advisory Committee held discussions on the expanded use of bioassay tests on point source and non-point source discharges and on ambient monitoring. These short-term tests include the Ames test for mutagenicity.

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- An EPA Workshop on the Ames Mutagenicity Test co-sponsored by the National Enforcement Investigations Center and the Environmental Monitoring and Support Laboratory-Cincinnati was held at the Federal Center, Denver, Colorado, December 5 and 6, 1979. Attendees included representatives of the EPA Regional Surveillance and Analyses Divisions, Air and Water Research Laboratories, and interested program offices. The Workshop determined the status of the Ames Mutagenicy Test in the Agency, explored anticipated use of the test in response to program needs, reviewed a draft of the test procedure used by NEIC to analyze complex waste mixtures, and considered standardization and quality assurance of the methodology.

- Following this workshop, the EPA Microbiological Methods Committee met in Denver and recommended that the NEIC draft procedure be modified based on attendees' suggestions and published as a developmental method.

The manual is recommended for use in enforcement, monitoring, and research. However, it is not intended to inhibit or prevent research and development.

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The author gratefully acknowledges the efforts of the staff of NEIC, EMSL-Cincinnati, HERL-RTP and certain EPA Regional offices for their support, editorial comments and reviews during the preparation of this document.

1. INTRODUCTION

There is increasing evidence that environmental mutagens are a major cause of cancer and genetic birth defects, and contribute to aging and heart disease. Humans are constantly exposed to mutagenic chemical contaminants in air, water, and food. Drinking water, municipal and industrial discharges, hazardous waste disposal sites and receiving waters must be monitored for mutagenic pollutants by systematic testing procedures and corrective actions taken when such substances are found. However, monitoring that uses complex biochemical or long-term animal tests is prohibitively expensive in time, manpower, and dollars.

Short-term tests to screen environmental wastes for mutagenicity are needed. One rapid screening test, the Salmonella/mammalian microsome mutagenicity assay (commonly known as the Ames test) has been investigated more thoroughly than others and proven reliable for identification of mutagenic and potentially carcinogenic substances.

Modified versions of the Ames test have been developed and used successfully with supporting chemical data by the EPA's NEIC in Denver, the Health Effects Research Laboratory, Research Triangle Park, and several Regions for air and wastewater characterization and health effects research.

Other EPA Regional and research laboratories and program offices with responsibility for toxic substances and hazardous materials have expressed an immediate need for the application of the Ames test in their activities. This manual was prepared to meet that need.

2. SUMMARY OF METHOD

The test system developed by Ames[1] has been widely used as a rapid screening procedure for the determination of mutagenic and potential carcinogenic hazards of complex environmental effluents and commercial products.

The assay uses five standard strains of histidine-requiring Salmonella mutants that revert after interaction with mutagens to the wild-type state (histidine-independence). The rate of reversion indicates the degree of mutagenicity. Relevancy of this *in vitro* bacterial test to carcinogenesis is increased by adding rat-liver homogenate to the test system. The rat-liver contains enzymes which perform several metabolic conversions similar to those of mammalian organs. Test sensitivity is enhanced by additional mutations in the test strains and by extrachromosomal resistance transfer factors in two of the strains.

3. SCOPE AND APPLICATION

The Ames test has been used successfully as a screening procedure to predict the mutagenic and potential carcinogenic hazards from pure compounds, commercial products and complex discharges[2,3,4,5].

3.1 ADVANTAGES

- The Ames test is simple, rapid, and relatively inexpensive.
- Positive test results reportedly show a high correlation (85-93%) with substances that produce tumors in laboratory animals[1,5,6,7].
- The test system detects relatively low concentrations of mutagens.
- The system has been tested and validated in several independent studies[5,6,7].

3.2 DISADVANTAGES

- Toxicity of complex mixtures may prevent measurement of mutagenic effects.
- Volatile organic compounds may be lost before measurement can be determined.
- Some non-mutagenic carcinogens, i.e. alkyl-halides, antibiotics, hormones, are not detectable in the test.
- The test does not consider pharmacokinetics involved in mutagenesis and carcinogenesis.

4. FACILITIES, EQUIPMENT, AND SUPPLIES

4.1 GENERAL REQUIREMENTS

Microbial assays for mutagenicity should be performed in a stationary laboratory. Generally, support equipment used in a typical water microbiology laboratory is suitable for use in the Ames test. This equipment includes good quality autoclaves, colony counters, sterilization ovens, incubators, waterbaths, water distillation systems, dishwashers, refrigerators, freezers, balances (analytical and top loading) and the usual pipettes and glassware for preparation of media and reagents. These items are described in the EPA Microbiological Methods Manual[2] and Standard Methods[3].

A general list of equipment, supplies, media and reagents (requirements for 20-30 samples) necessary for environmental mutagenesis testing is presented below:

4.2 MAJOR EQUIPMENT

	ITEM	NO.	SUGGESTED SOURCE*
a.	Membrane filtration system, for sterilization of heat-labile materials	2	Millipore Corp.
b.	Quebec Colony Counter, darkfield, with electronic register	1	Scientific Products, Inc.
c.	Automated Colony Counter, (optional)	1	New Brunswick Scientific Co., Inc.
d.	Petri dish Filler/Stacker, (optional)	1	New Brunswick Scientific Co., Inc.
e.	Laminar-Flow Biological Safety and Fume Hood, equipped with total exhaust system	1	Contamination Controls, Inc.

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* The mention of trade names or commercial products does not constitute endorsement or recommendation for use.

f. "Shaker-in-the-Round", for extraction procedure	2	Scientific Products, Inc.
g. Dri-block Heater, to hold 13 x 100 mm test tubes with accessories	2-3	Scientific Products, Inc.
h. Evaporator, Rotovapor R/A, with accessories	2	Van Waters and Rogers Scientific Co., Inc.
i. Shaker Waterbath, for culturing test strains	1	New Brunswick Scientific Co., Inc.
j. Waterbath, for tempering media	1	Scientific Products, Inc.
k. Ultra-freezer (-80°C), 12.5 cu ft	1	Revco Scientific Co.
l. Explosion-proof Refrigerator, 3.5 cu ft, for storage of standard diagnostic mutagens, equipped with security lock	1	Scientific Products, Inc.
m. Mechanical Pipetting Device	2-3	Bellco Glass, Inc.
n. Bag Sealing Device, for sealing petri dishes in plastic bags	1	Sears-Roebuck & Co.

4.3 EXPENDABLE EQUIPMENT AND SUPPLIES

ITEM	NO.	SUGGESTED SOURCE
a. Micro-volume Pipettes 1 µl-1000 µl volume, with sterile disposable tips	2-3	Cole-Parmer Instrument Co.
b. Sealable Plastic Bags, for sealing petri dishes	4-5 cases of 500	Sears-Roebuck & Co.
c. Surgical Gloves, latex, disposable	1-2 cases of 500	Pharma Seal Laboratories
d. Petri Dishes 15 x 100 mm, gamma-irradiation sterilized, disposable	1-5 cases of 500	Falcon Plastics, Inc.
e. Test Tubes, 13 x 100 mm, disposable	4-5 cases of 1000	Bellco Glass, Inc.

f.	Caps, for 13 x 100 mm test tubes, color coded	1 case of 1000 ea	Bellco Glass, Inc.
g.	Test Tubes, 20 x 125 mm, screw-capped	1 case of 500	Corning Glass, Inc.
h.	Reagent Bottles, screw-capped for storage of media and reagents, 100, 200, 300, 400, 500 ml volume	1 doz. ea	Bellco Glass Inc.
i.	Pipettes, TD, disposable, sterile, glass, 1 ml, 2 ml, 5 ml and 10 ml volumes	1 case of 1000 ea	Scientific Products Co.
j.	Separatory funnels, 2000 ml volume for extraction procedure	6-8	Kimball Glass Co.
k.	Round-bottom flasks, 50, 100, 1000-ml volumes, to fit evaporator rotovapor devices	1 doz. ea	Kimball Glass Co.
l.	Erlenmeyer Flasks, 250, 500, 1000, 2000 ml volumes	1 doz. ea	Kimball Glass Co.
m.	Sample Bottles, amber-glass, 1-gal volume	3 doz.	Kimball Glass Co.
n.	Kuderna-Danish Evaporative Concentrator, (flasks, 3-ball Schneider columns, graduated concentrator tubes)	5-10 ea	Kontes Co.

4.4 REAGENTS

	ITEM	AMOUNT	SUGGESTED SOURCE
a.	Ampicillin, diagnostic reagent (special preparation, high purity)	5g	Bristol Laboratories
b.	D-Biotin (M.W. 244.31)	5 g	Eastman Kodak Co. (# 14635)
c.	Citric Acid	500 g	J. T. Baker Chemical Co.
d.	Crystal-violet	10 g	Difco Laboratories, Inc.
e.	Dextrose	500 g	Difco Laboratories, Inc.

f. Methylene Chloride (Dichloromethane) distilled in glass	5 gal	Burdick & Jackson Laboratories
g. Dimethyl Sulfoxide (DMSO) Spectrophotometric quality	1 gal	Matheson, Coleman & Bell (# MX1454)
h. Dipotassium Hydrogen Phosphate (K_2HPO_4)	1 lb	Mallinckrodt Chemical Works
i. Disodium Hydrogen Phosphate ($Na_2HPO_4 \cdot 7H_2O$)	1 lb	Mallinckrodt Chemical Works
j. Glucose-6-Phosphate (M.W. 282.1) anhydrous	10 g	Sigma Chemical Co. (# G7879)
k. Hydrochloric Acid (HCl)	9 lb	J. T. Baker Chemical Co.
l. L-Histidine (M.W. 192.7) anhydrous	10 g	Sigma Chemical Co. (# H8125)
m. Magnesium Chloride ($MgCl_2 \cdot 6H_2O$)	1 lb	J. T. Baker Chemical Co.
n. Magnesium Sulfate ($MgSO_4 \cdot 7H_2O$)	1 lb	J. T. Baker Chemical Co.
o. Nicotinamide Adenine Dinucleotide Phosphate (M.W. 765.4) anhydrous	10 g	Sigma Chemical Co. (#N0505)
p. Potassium Chloride (KCl)	1 lb	J. T. Baker Chemical Co.
q. Sodium Ammonium Phosphate ($NaNH_4PO_4 \cdot H_2O$)	1 lb	J. T. Baker Chemical Co.
r. Sodium Chloride (NaCl)	1 lb	J. T. Baker Chemical Co.
s. Sodium Dihydrogen Phosphate ($NaH_2PO_4 \cdot H_2O$)	1 lb	Fisher Scientific Co.
t. Sodium Hydroxide (NaOH)	1 lb	J. T. Baker Chemical Co.
u. Sodium Sulfate (Na_2SO_4) anhydrous	5 lb	J. T. Baker Chemical Co.

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4.5 PREPARED MEDIA

	ITEM	AMOUNT	SUGGESTED SOURCE
a.	Ampicillin, "Dispens-o-Discs", 10 µg	250	Difco Laboratories (#6363)
b.	Purified Agar (Oxoid, #L28)	5 lb	K. C. Biological Inc.
c.	Nutrient Broth (Oxoid, #2)	5 lb	K. C. Biological Inc. (# CM67)
d.	Rat Liver Homogenate		

Rat Liver Homogenate (RLH) is the supernatant from 9000 x g minced rat-liver, using animals induced with Aroclor-1254 five days before sacrifice. It is recommended that laboratories purchase the prepared material from commercial biological supply companies or from private laboratories performing mutagen research.* Quick-freeze with dry ice immediately after preparation, and store at -80°C in 2 ml, plastic vials.

5. PREPARATION OF MEDIA AND REAGENTS

5.1 MINIMAL AGAR

Composition

Solution A:		Solution B:	
Purified Agar (Oxoid, #L28)	5.0g	Purified Agar	5.0 g
Distilled Water	500 ml	Dextrose	20.0 g
		Distilled Water	480 ml

Preparation

Dissolve the contents of each flask by heating in a boiling water bath. Autoclave 15 minutes at 121°C (15 lb pressure). Remove from the autoclave and cool to 45°C in a water bath. Add 20 ml of Vogel-Bonner Medium "E" (50X) solution (prewarmed to 45°C) to Solution B. Combine the two solutions and dispense 30 ml/plate into 100 x 15 mm petri dishes.

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* Rat Liver homogenate is available from the AMC Cancer Research Center and Hospital c/o Dr. Elias Balbinder, 6401 W. Colfax Ave., Lakewood, CO 80214; or from Litton Bionetics, 5516 Nicholson Lane, Kensington, MD 20795.

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5.2 VOGEL-BONNER MEDIUM "E" SOLUTION (50X)

Composition

$MgSO_4 \cdot 7H_2O$	10.0 g
Citric Acid	100.0 g
Dipotassium Hydrogen Phosphate (K_2HPO_4, anhydrous)	500.0 g
Sodium Ammonium Phosphate ($NaNH_4HPO_4 \cdot 4H_2O$)	175.0 g
Distilled Water	670 ml

Preparation

Slowly dissolve the ingredients. Solution will require warming. Final volume is 1 liter. 'Autoclave 15 minutes at 121°C (15 lb pressure). Store in refrigerator.

5.3 NUTRIENT AGAR

Composition

Purified Agar (Oxoid, #L28)	10.0 g
Lab-lemco powder	10.0 g
Peptone	10.0 g
Sodium Chloride (NaCl)	5.0 g
Distilled Water	1000 ml

Preparation

Heat in a boiling water bath to dissolve completely. Autoclave 15 minutes at 121°C (15 lb pressure). Remove from autoclave and cool to 45°C in water bath. Dispense 30 ml/plate into 100 x 15 mm petri dishes.

5.4 NUTRIENT BROTH (Oxoid #2)

Composition

Lab-lemco Powder	10.0 g
Peptone	10.0 g
Sodium Chloride (NaCl)	5.0 g
Distilled Water	1000 ml

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Preparation

Dissolve contents completely. Dispense into 20 x 125 mm screw-capped tubes, 10 ml/tube. Autoclave 15 minutes at 121°C (15 lb pressure).

5.5 TOP AGAR

Composition

Purified Agar (Oxoid #L28)	1.2 g
Sodium Chloride (NaCl)	1.0 g
Distilled Water	200 ml

Preparation

Heat in a boiling water bath to dissolve completely. Dispense into screw-capped bottles, 200 ml/bottle. Autoclave 15 minutes at 121°C. Store in refrigerator. Before use, melt top agar in autoclave or microwave oven, cool to 45°C and add 20 ml histidine/biotin solution/200 ml (prewarmed to 45°C).

5.6 L-HISTIDINE·HCl (0.1 M)

Composition

L-Histidine·HCl	1.927 g
Distilled Water	100 ml

Preparation

Sterilize by filtration through a 0.22 μm membrane filter. Store in refrigerator.

5.7 D-BIOTIN (0.5 mM)

Composition

D-Biotin	0.0122 g
Distilled Water	100 ml

Preparation

Sterilize by filtration through a 0.22 μm membrane filter. Store in refrigerator.

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5.8 L-HISTIDINE·HCl (0.5 mM)/D-BIOTIN (0.5 mM) SOLUTION

Composition

L-Histidine·HCl	0.0240 g
D-Biotin	0.0305 g
Distilled Water	250 ml

Preparation

Sterilize by filtration through a 0.22 μm membrane filter. Store in refrigerator.

5.9 SALT SOLUTION

Composition

1.65 M Potassium Chloride (KCl)	61.42 g
0.4 M Magnesium Chloride ($MgCl_2 \cdot 6H_2O$)	40.66 g
Distilled Water	500 ml

Preparation

Autoclave to sterilize. Store in refrigerator.

5.10 SODIUM PHOSPHATE BUFFER

Composition

Sodium Dihydrogen Phosphate ($NaH_2PO_4 \cdot H_2O$)	4.14 g
Disodium Hydrogen Phosphate ($Na_2HPO_4 . 7H_2O$)	21.45 g
Distilled Water	550 ml

Preparation

Dissolve the dibasic salt in 400 ml H_2O, and the monobasic salt in 150 ml H_2O. Add dibasic solution to monobasic solution. Adjust pH to 7.4; autoclave to sterilize. Store in refrigerator.

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5.11 NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE (0.1 M)

Composition

NADP	7.65 g
Distilled Water	100 ml

Preparation

Sterilize by filtration through a 0.22 µm membrane filter. Store in 13 x 100 mm stoppered test tubes in 2 ml volumes in freezer at -20°C.

5.12 GLUCOSE-6-PHOSPHATE (1 M)

Composition

G-6-P	2.82 g
Distilled Water	10 ml

Preparation

Sterilize by filtration through a 0.22 µm membrane filter. Store in 13 x 100 mm stoppered test tubes in 0.3 ml volumes in freezer at -20°C.

5.13 RAT LIVER ENZYME MIX

On the day of the test, using aseptic technique, the following ingredients are combined in a sterile graduate cylinder which has been placed in an ice water bath. The rat liver enzyme mix must be chilled in an ice bath during the test.

Rat Liver Homogenate	2.0 ml
Salt Solution	1.0 ml
G-6-P	0.25 ml
NADP	2.0 ml
Sodium Phosphate Buffer	25.0 ml
Sterile Distilled Water	19.75 ml

5.14 AMPICILLIN SOLUTION (8 mg/ml)

Composition

Ampicillin trihydrate	0.8 g
Sodium Hydroxide (0.02 N)	100 ml

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Preparation

Sterilize by filtration through a 0.22 μm membrane filter. Store in refrigerator.

6. SALMONELLA TEST STRAINS

6.1 TEST SYSTEM

The Ames test is based on the use of five standard strains of *Salmonella typhimurium* containing a specific mutation in the histidine operon. These genetically altered strains cannot grow in the absence of histidine; when they are placed in a histidine-free medium, only those cells that revert spontaneously to the wild-type condition (histidine-independence) are able to form colonies. The spontaneous mutation rate of each strain is relatively constant, but if a chemical mutagen is added to the medium the mutation rate is increased significantly.

The sensitivity of the *Salmonella* test strains has been enhanced by the introduction of two additional mutations, labeled *uvrB* and *rfa*. The *uvrB* gene mutation results in an error-prone repair of DNA damage caused by certain chemical or physical agents. The *rfa* (deep rough) character allows increased cell permeability and greater penetration of chemical mutagens into the bacterial cell, due to a defective lypopolysaccharide layer in the cell wall.

Certain chemicals will not display mutagenic activity unless they are converted metabolically to an active form. The Ames test employs rat-liver enzymes to simulate mammalian metabolism. Liver microsomes are added to the test chemicals to detect the intermediate metabolites that are often potent mutagens.

6.2 TEST STRAINS

The five standard *Salmonella* test strains used are described in the table below. TA 1535 has a missense mutation in *his G46* and is used to detect mutagens that cause base-pair substitutions (e.g., replacement of one nucleotide base pair in DNA by another) Strains TA 1537 and TA 1538 are sensitive to mutagens that cause the frame shift mutations (e.g., addition or removal of base pairs) his C3076 and his D3052, respectively.

Strains TA 100 and TA 98 are daughter strains of TA 1535 and TA 1538, respectively; these contain the resistance transfer factor plasmid (R-factor) pKM 101. The R-factor increases sensitivity to certain mutagens, possibly through error-prone repair.

GENOTYPE OF FIVE SALMONELLA TEST STRAINS
USED FOR GENERAL SCREENING IN MUTAGEN TESTING

Strain	Mutation	Repair	LPS	R-Factor
TA 1535	Missense Mutation his G46	uvrB	rfa	-
TA 100	Missense Mutation his G46	uvrB	rfa	pKM 101
TA 1537	Frameshift Mutation his C3076	uvrB	rfa	-
TA 1538	Frameshift Mutation his D3052	uvrB	rfa	-
TA 98	Frameshift Mutation his D3052	uvrB	rfa	pKM 101

The five Salmonella strains used in the Ames test may be obtained from Dr. Bruce N. Ames, Biochemistry Department, University of California, Berkeley, California 94720. The bacteria are impregnated onto small absorbent pads.

6.3 REGENERATION AND STORAGE OF TEST STRAINS

a. Upon receipt of the strains, streak each pad onto individual nutrient agar plates. For strains TA 98 and TA 100, use plates containing 25 μg/ml ampicillin solution*. Incubate agar plates at 37°C ± 0.5° C for 24 hr.

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* Ampicillin (0.1 ml of an 8 mg/ml solution) may be applied directly to the agar plates using a sterile, glass spreader. Prepare the plates one day in advance to allow the antibiotic solution to diffuse uniformly throughout the agar.

b. After streaking, place each pad into a test tube containing 10 ml of nutrient broth. For TA 98 and TA 100 add 25 µg/ml ampicillin solution.

c. Incubate plates at 37°C for 24 hr. Incubate broth cultures at 37°C overnight, shaking constantly (16 hr maximum).

d. Frozen permanent stocks are prepared in duplicate as follows: Pipette 0.8 ml of nutrient broth culture into a 2 ml, sterile, glass, screw-capped vial with teflon liner. Add 0.07 ml dimethyl sulfoxide (DMSO).

e. Quick-freeze vials in dry ice and place in freezer at -80°C.

f. Store agar plate cultures at 4°C until the broth cultures are checked for proper function and determined to be satisfactory. Use one culture of each strain to prepare a master plate (Section 7). Open the remaining permanent culture only to regenerate the frozen stocks.

7. ANALYTICAL PROCEDURES

7.1 PREPARATION OF MASTER PLATES[4]

To avoid cell death by continued freezing and thawing of the permanent stocks, prepare master plates. These plates may be stored at 4°C for approximately six weeks if they are sealed to prevent dehydration.

a. Prepare fresh broth cultures by scraping the frozen broth cultures with a sterile wooden applicator stick and inoculate nutrient broth tubes. For strains TA 98 and TA 100, the broth contains 25 µg/ml ampicillin. Incubate, shaking constantly (100 rpm), at 37°C overnight (16 hr maximum).

b. Prepare minimal agar plates by spreading 0.1 ml of sterile 0.1M L-histidine on the surface of each plate (for TA 98 and TA 100 plates contain 25 μg/ml ampicillin).

c. Allow the histidine solution to be absorbed into the agar.

d. Spread 0.1 ml of sterile 0.5 mM biotin on the surface of each plate and allow the solution to be absorbed into the medium.

e. Using a sterile loop, streak each plate with the appropriate test strain so that isolated colonies are obtained.

f. Incubate plates at 37°C for 48 hr.

g. Seal each plate by wrapping with tape or parafilm; store at 4°C.

h. Retain nutrient broth culture to observe test strain function.

7.2 TEST STRAIN FUNCTION

Routinely check test strains for reversion rates and proper genetic markers.

7.2.1 Histidine Requirement

a. For each strain prepare two minimal agar plates by coating each plate with 0.1 ml of sterile 0.5 mM biotin. Spread 0.1 ml of sterile 0.1 M histidine onto one of the plates.

b. Apply a single cross-streak of broth culture to the plates with each test strain. Incubate plates at 37°C for 24 hr.

Each strain should show growth on the plate containing histidine; no growth should occur on the plate containing biotin.

7.2.2 Crystal-violet (rfa character)/Ampicillin Sensitivity (R-factor)

a. Pipet 0.1 ml broth culture into 2 ml top agar (with histidine/biotin solution added). Pour mixture onto a minimal agar plate and allow to solidify. Using sterile tweezers, place a filter paper disc (soaked with a 10% solution of crystal-violet) off-center of the plate.

b. Place a 10 μg ampicillin impregnated disc (Difco #6363 "Dispens-o-Disc") off-center, opposite the crystal-violet disc. Use a separate plate for each test strain. Incubate 24 hours at 37°C.

All test strains should have a zone of growth inhibition (approximately 14 mm diameter) around the crystal-violet, indicating the presence of the rfa mutation. Strains TA 1535, TA 1537 and TA 1538 should show a zone of growth inhibition around the ampicillin disc. Strains TA 98 and TA 100 (containing the R-factor) are not inhibited by the ampicillin [Figures 1 and 2].

7.2.3 UV Sensitivity (uvrB-deletion)

Cross-streak each test strain on a nutrient agar plate. Irradiate one-half of the plate under a 15 Watt germicidal lamp at a distance of 33 cm (13 in) for 8 sec. Incubate plates at 37°C for 18 to 24 hr.

All strains should retain the uvrB-deletion and demonstrate growth on the unexposed portion of the plate only [Figure 3].

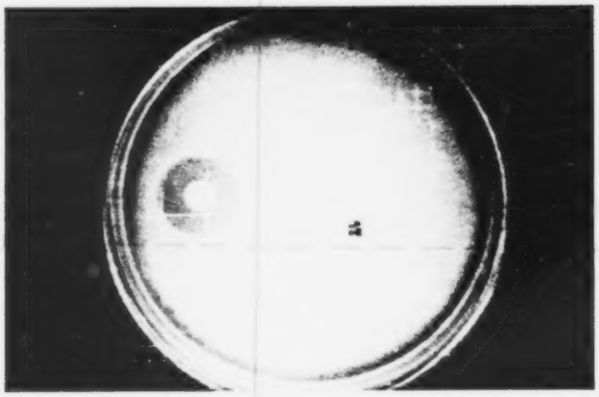

Figure 1. Test Strain TA98. Crystal-violet sensitivity (rfa-character) and ampicillin resistance (R-factor).

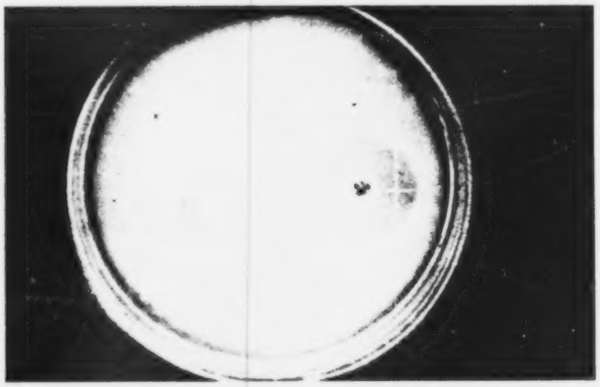

Figure 2. Test Strain TA1537. Crystal-violet sensitivity (rfa-character) and ampicillin sensitivity (does not contain R-factor).

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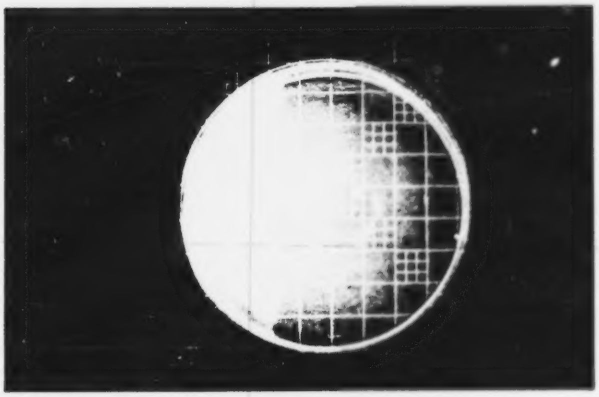

Figure 3. Test Strain TA98, Growth inhibition after exposure to UV light (**uvrB**-deletion).

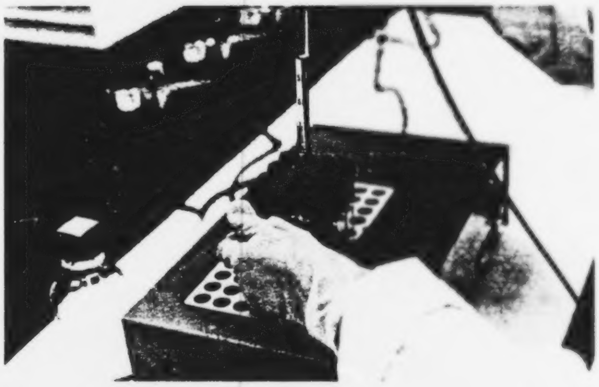

Figure 4. Filling 13 × 100 mm culture tubes with 2 ml top-agar (heating block set at 45° C).

7.2.4 Spontaneous Reversion

Spontaneous reversion control tests should be performed in duplicate during each test. Results should be maintained as part of the permanent quality control record for each study.

a. Prepare top agar (Section 5). Melt prepared top agar in autoclave for three minutes at 121°C, or melt the agar in a microwave oven. Cool to 45°C in a waterbath.

b. Prepare histidine/biotin solution (Section 5). Warm histidine/biotin solution in 45°C waterbath. Add 20 ml histidine/biotin solution per 200 ml top agar.

c. Preset heating block containing sterile, capped, 13 x 100 mm culture tubes at 45°C. Pipet 2 ml molten top agar into each tube [Figure 4].

The following steps should be performed under a laminar-flow hood.

d. Add 0.1 ml broth culture of each test strain to each of two duplicate tubes.

e. Add 0.5 ml rat liver enzyme mix (RLE), if RLE mix is being used in the mutagenesis assay.

f. Rotate each tube rapidly between the palms to mix the material [Figure 5]. (A vortex-mixer may be used if mixing is performed gently to prevent bubble formation in the top agar.)

g. Pour the contents of each tube onto the surface of a minimal agar plate [Figure 6]. Gently tilt and rotate each plate to spread the top agar uniformly over the surface of the minimal agar.

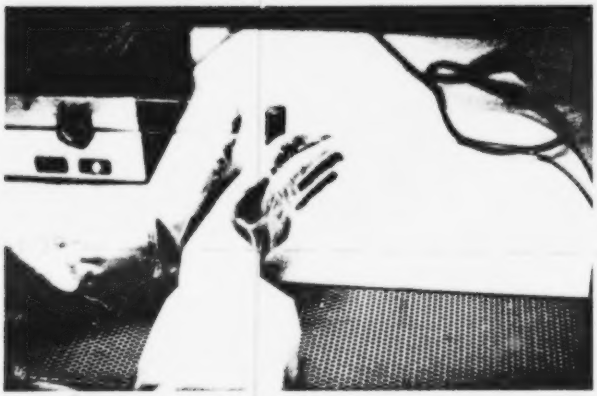

Figure 5. Mixing top-agar by rotating tube between palms.

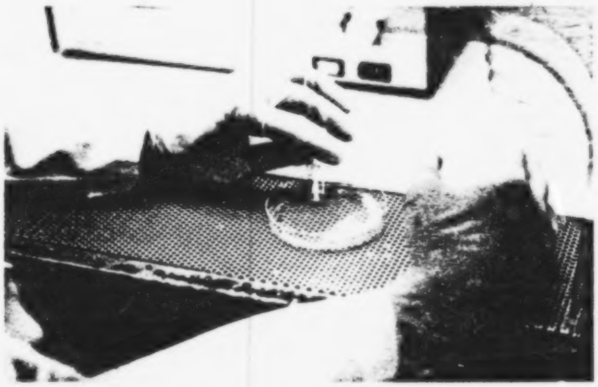

Figure 6. Pouring top-agar onto minimal-agar plate.

The organisms should remain at 45°C for no longer than 2-min to avoid cell death. Also, RLE mix cannot be subjected to 45°C for more than a few seconds. Therefore, the above steps should be performed as quickly as possible. If the top agar hardens in mid-operation, a stippled agar surface will occur, which may cause difficulties in counting the colonies.

h. Set each plate, with lid slightly ajar, on a flat surface to dry for several minutes. Cover the plate, seal in sealable plastic bag [Figure 7] and incubate at 37°C for 48 hr in an inverted position.

i. Count the number of revertant colonies. Use a dissecting microscope to note the presence of a light "lawn" of background growth which is due to the trace amount of histidine in the top agar.

Expected Spontaneous Revertants for Each Strain

Strain	No. Of Colonies
TA 1535	10-35
TA 1537	3-15
TA 1538	15-35
TA 100	120-200
TA 98	30-50

Revertant rates may be slightly higher on plates containing RLE mix due to the histidine present in the homogenate. Each laboratory should establish an acceptable range of spontaneous revertants per plate to determine if the strains are responding properly.

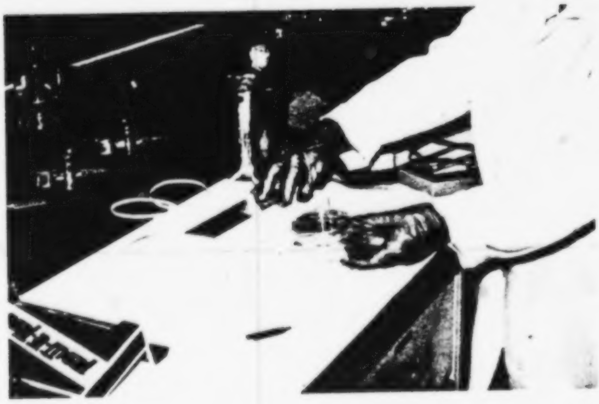

Figure 7. Sealing agar plate in plastic bag.

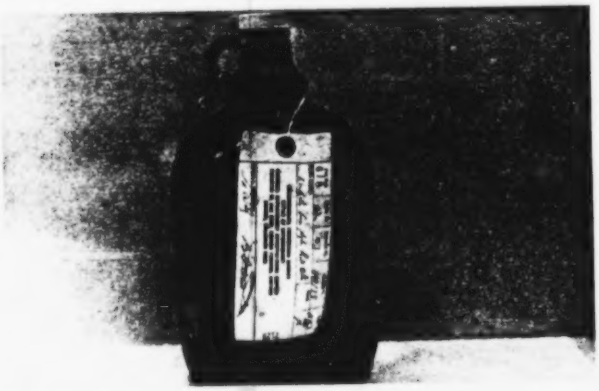

Figure 8. Example of a 1-gallon amber-glass sample bottle equipped with teflon-lined cap.

7.3 COLLECTION AND PREPARATION OF SAMPLES

7.3.1 Sample Collection

a. Collect a gallon sample from each water and wastewater source,* in a clean, amber-glass bottle with a teflon lined cap [Figure 8]. To assure that wastewater samples are representative, collect them on a flow-composite basis over a 24-hr period. Collect time-proportional composit samples where discharges are intermittent. Grab samples are acceptable from wells, pools, and lakes.

b. Ice the samples during collection and transport, and refrigerate at 0-4°C during storage. Samples should not be held longer than 7 days between collection and extraction. DMSO extracts may be held for 30 days. Record all sampling information as a permanent laboratory record [Figure 9].

7.3.2 Extraction and Concentration of Samples

Base-Neutral Extraction

Adjust the sample to pH 12 with 6N NaOH solution. Divide the sample into four equal portions. Record the total volume and transfer each portion into a 2-liter separatory funnel. Extract as follows:

a. Add 100 ml of methylene chloride (dichloromethane) to a sample portion into a 2-liter separatory funnel.

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* Samples suspected of containing residual chlorine should be treated with 0.8 ml of a 10% solution sodium thiosulfate/liter of sample. This concentration will neutralize approximately 15 mg/l residual chlorine.

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Figure 9

SAMPLE DATA FORM

Station No.	Station Description	Date	Time	Seq No.	Grab	Comp	Samplers	Remarks (Preservative)	Signed In By

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b. Shake the mixture for 2 min and place in a ring stand until the phases separate. If an emulsion develops, centrifuge at 2,000 rpm for 2 to 5 min and return the mixture to the separatory funnel.

c. Remove the aqueous fraction and retain for further extraction. Save the solvent fraction.

d. Extract the aqueous fraction two additional times using 60 ml of methylene chloride. Combine the solvent fraction of the sample portion.

e. Retain the aqueous fraction for acid extraction.

f. Repeat Steps 'a' through 'e' for the other three portions of the sample.

g. Combine the solvent fractions from the entire sample (approximately 880 ml) and pour through a drying column containing 7 to 10 cm (3 to 4 in) of anhydrous sodium sulfate, which has been pre-rinsed with 40 to 50 ml of methylene chloride.

h. Add approximately 500 ml of the solvent extract to a 1000-ml Kuderna-Danish (K-D) flask equipped with a 3-ball Schneider-column and a 10 ml graduated concentrator tube.

i. Place 1 or 2 boiling chips into the K-D flask and evaporate in a steam bath (adding the remaining solvent extract in stages) to a final volume of approximately 500 ml.

j. Add 150 ml of acetone to the concentrated solvent extract, and continue evaporation to a volume of 5 ml.

k. Add acetone to bring volume to 10 ml and mix well. Retain 2.5 ml for trace organic chemical analyses.

l. Add 10 ml of DMSO to the remaining 7.5 ml of sample extract and mix well. Evaporate the extract to 10 ml in a roto-evaporator at 65°C.

m. Take up the total residue in 25 ml of DMSO to a total volume of 35 ml. Record all extraction information [Figure 10].

A flow diagram of the extraction procedure is shown in Figure 11. Label the sample extract, and store at 4°C in a secured, explosion-proof refrigerator until assayed by the Ames procedure.

Acid Extraction

n. Acidify the aqueous fraction from Step e to pH 2 with 6N HCL. Repeat the extraction procedure.

o. Discard the aqueous portion of the sample after the final extraction.

p. Collect a gallon sample of mutagen-free, laboratory-pure water in a clean, amber-glass bottle. Repeat Steps 'a' through 'o' on this water blank sample. (See Quality Control Procedures, Section 8).

q. Record all extraction information [Figure 10].

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Figure 10

EXTRACTION PROCEDURE DATA FORM

Sta. No	Seq. No	Date Sampled	OPERATION PERFORMED								
			pH Adjust.	Int.	Solvent Extract'n	Int.	Emuls'n. Removed	Int.	Sample Conc.	Int.	Signature/ Date

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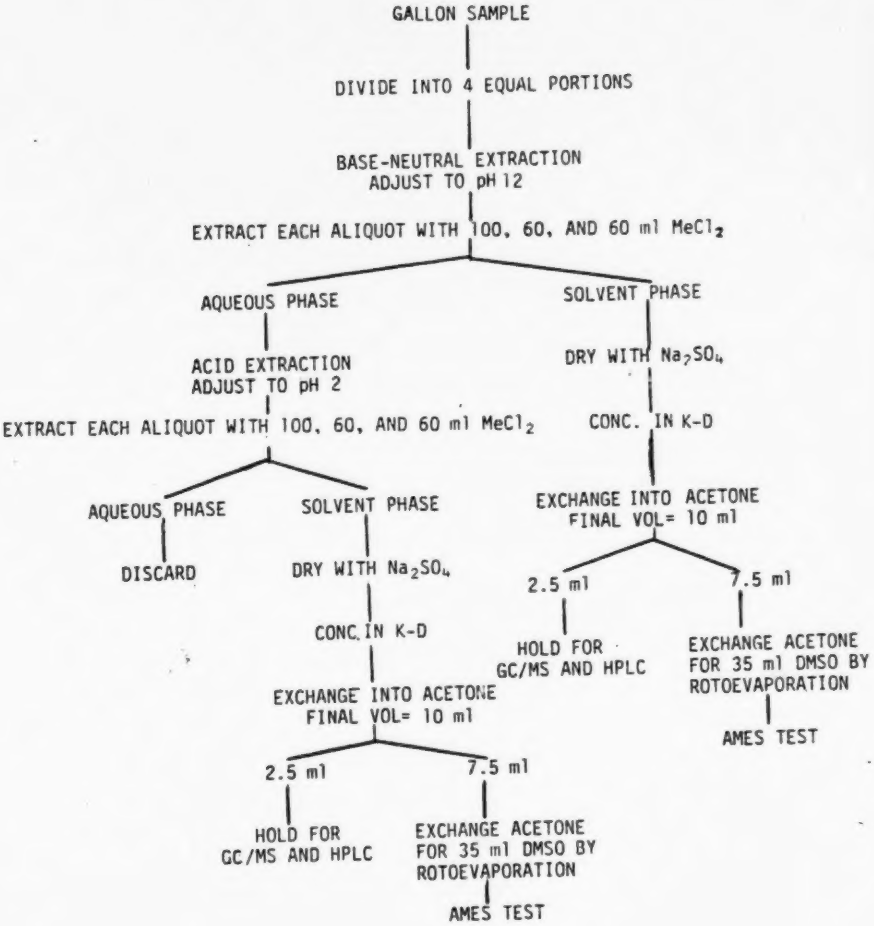

Figure 11

Extraction Procedure for Environmental Water Samples

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7.4 MUTAGENESIS ASSAYS

7.4.1 Plate Incorporation Procedure - Primary Screen [Figure 12]

a. On the day prior to performance of the assay, pick single isolated colonies from the master plates and inoculate tubes (20 x 125 mm) containing 10 ml nutrient broth with the five tester strains.*

b. Incubate tubes with constant agitation for 16 hr at 37°C.

c. Prepare top agar as outlined in Section 5. Melt prepared top agar in autoclave for 3 min at 121°C, or melt the agar in a microwave oven. Cool to 45°C in a waterbath.

d. Prepare histidine/biotin solution as outlined in Section 5. Place histidine/biotin solution in 45°C waterbath to warm. Add 20 ml histidine/biotin solution per 200 ml top agar.

e. If possible, prewarm minimal agar plates to 37°C before inoculation.

f. Preset heating block containing sterile 13 x 100 mm culture tubes to 45°C. Pipet 2 ml molten top agar into each tube.

Perform the following steps under a laminar-flow hood; wear fully fastened laboratory coat and surgical gloves.

g. Add 0.1 ml broth culture (from Step 'b') [Figure 12] of the desired test strain to each tube corresponding to number of dilutions of extract (suspected mutagen) to be tested. Broth cultures should be placed in an ice bath

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* Do not add ampicillin to broth cultures for strains TA 98 and TA 100 during the mutagenesis assays; the antibiotic can sometimes cause excessive bacterial killing if it is concentrated in test tube cultures.

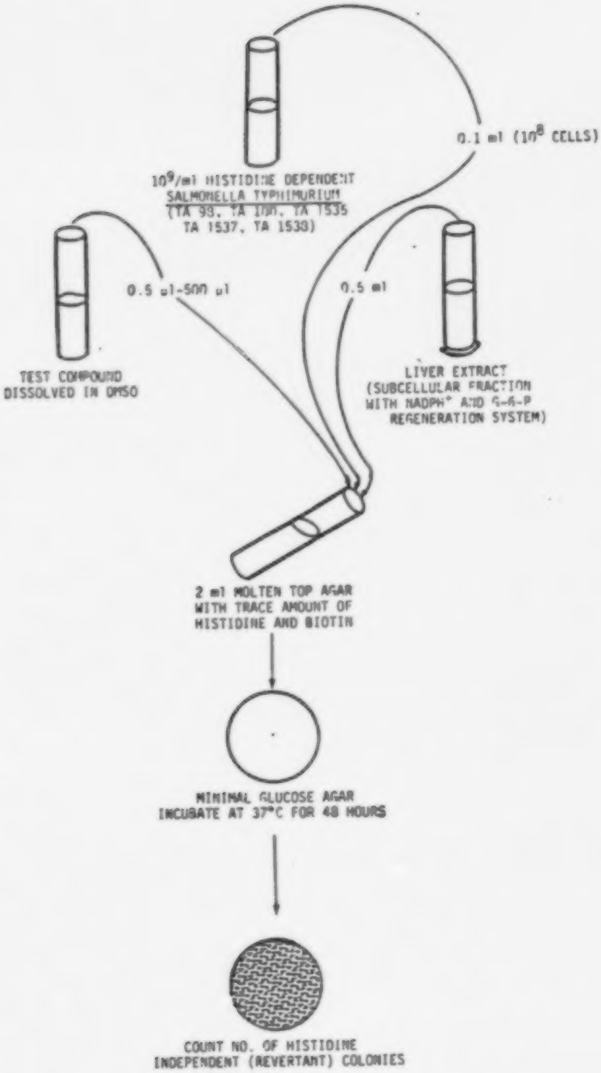

Figure 12
Agar Plate Incorporation Procedure

for the duration of the test. Remember that upon inoculation the organisms must not remain at 45°C for more than 2 min. Inoculate tubes singly to avoid prolonged exposure to 45°C temperature

h. Add an appropriate concentration of test material to each tube inoculated with culture. Suggested volumes of extract include 500 µl, 250 µl, 100 µl, 50 µl and 10 µl for each test strain.

i. Rotate each tube rapidly between palms to thoroughly mix the materials. Alternately, a Vortex-mixer may be used, if mixing is performed gently to prevent bubble formation in the top agar.

j. Pour the contents of each tube onto the surface of a minimal agar plate. Gently tilt and rotate each plate to spread the top agar uniformly over the surface of the minimal agar.

k. Set each plate, with lid slightly ajar, on a flat surface to dry for several minutes. Cover the plates, seal in sealable plastic bags, and incubate at 37°C for 48 hr in an inverted position. Count the number of revertant colonies [Figure 13].

l. Initially, perform the assay without RLE mix. If mutagenic activity is observed, determine a dose-response with all active strains [Figure 14]. Then terminate the test. However, if mutagenic activity is not observed, repeat the above steps adding RLE mix after Step 'h'.

m. Add 0.5 ml of RLE mix to each tube. Immediately after the RLE mix is added, quickly rotate the tube between the palms and pour onto the surface of the minimal agar plate (Steps 'i' and 'j'). Allow only a few seconds for the addition of RLE mix. Proceed with Step 'k'.

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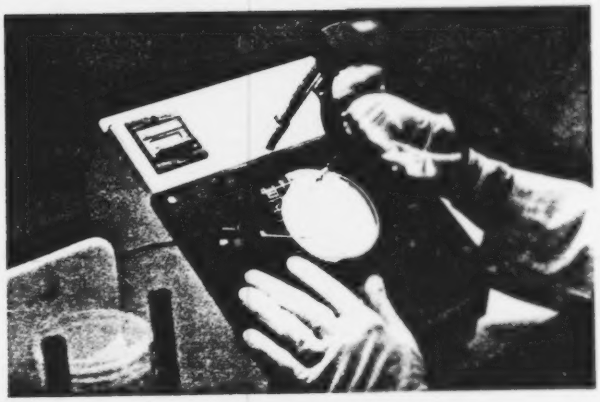

Figure 13. Counting revertant colonies using digital, Quebec Colony Counter.

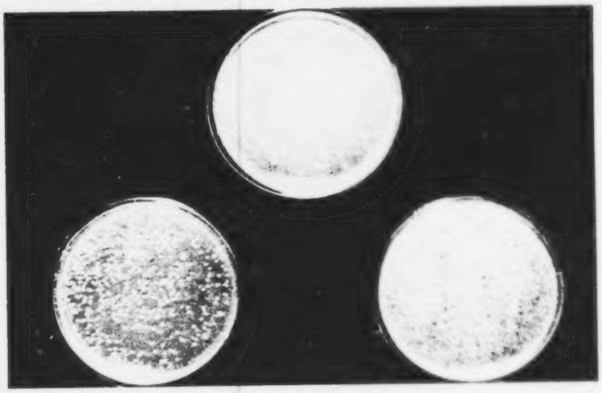

Figure 14. Dose-response effect of mutagenic material on Test Strain TA1537 (*top*–50 μl, *left*–100 μl, *right*–200 μl of concentrated waste extract).

7.4.2 Plate Incorporation Procedure - Dose Response

If increased numbers of revertants are noted, repeat all steps of the primary screen in duplicate (preferably triplicate) using additional doses of test material, with all active strains. For example, if the 250 µl concentration shows mutagenic activity, volumes of choice might include 100 µl, 150 µl, 200 µl, 250 µl, 300 µl, 350 µl, 400 µl, 450 µl, to establish a dose-response. Proper test aliquots must be selected independently for each sample tested.

Perform control plates (at least duplicates) for each test strain.

Use a dissecting microscope to examine the background lawn.

The background growth or lawn is a result of the trace amount of histidine present in the top agar. If the substance is toxic, massive cell death will occur and the background lawn will be sparse or absent compared to control plates. The sparse growth is a result of more histidine being available to the surviving bacteria. Survivors undergo more cell division and appear as small colonies which can be mistaken for revertants; consequently, the presence or absence of a normal lawn must be noted.

To check for histidine requirement, pick randomly selected revertant colonies from plates showing mutagenicity and streak onto a minimal agar plate.

All positive mutagenic responses should be verified by a repeatable assay.

7.4.3 Spot Test

The spot test is a simple method to determine if a compound is mutagenic qualitatively. The test is particularly useful to rapidly screen large numbers of compounds in a short time. Also, the spot test may be used to determine an approximate concentration range of suspected mutagen to employ in the plate incorporation assay. Results of the spot test are not conclusive; the test should be used only as an adjunct to the agar plate incorporation procedure.

The spot test can only be used for analyses of water soluble chemicals which are readily diffusible in agar. Also, it is much less sensitive than the standard agar plate incorporation procedure, because fewer bacteria are exposed to the test material at any dose level.

a. Prepare cultures, media, reagents and equipment (Steps 'a' through 'j' as in the Primary Screen assay, 7.4.1); but omit the test chemical. Perform the test both with and without 0.5 ml of rat liver enzyme mix added to the top agar.

b. After the top agar has hardened, quickly add the suspected mutagen to the agar surface in one of the following ways: (1) as crystals (for pure compounds), (2) as a 10 µl microdrop, or (3) as a 6 mm sterile filter paper disc saturated with test chemical.

c. Seal the plates in plastic bags and incubate at 37°C for 48 hr in an inverted position.

d. Perform all procedures at least in duplicate (preferably triplicate).

e. Prepare a positive control by adding a known diagnostic mutagen.

f. Prepare a negative control by adding a non-mutagenic crystal, a 10 μl microdrop of sterile distilled water, or a blank 6 mm sensi disc saturated with sterile distilled water.

g. Read and record results.

A positive result is usually indicated by a large increase in colonies located in a circular position away from the spot [Figure 15]. If the mutagen is non-toxic, colonies may develop directly adjacent to the spot.

Always confirm definitive mutagenicity by demonstrating a repeatable dose-response effect with the standard agar plate incorporation procedure.

7.4.4 Preincubation Assay

The most widely used and successful modification to the standard Ames test is the preincubation assay described by Yahagi[8]. This procedure is sometimes useful as an alternate test, if the test material is toxic, or in cases where results of the standard plate assay are inconclusive, e.g., detection of nitrosoamines. Preincubation tests are performed at least in duplicate.

a. On the day before performing the assay, inoculate from the master plates 20 x 125 mm tubes containing 10 ml nutrient broth with the five tester strains.

b. Incubate tubes with constant agitation for 16 hr at 37°C.

c. Prepare top agar as outlined in Section 5. Melt prepared top agar in autoclave for 3 min at 121°C, or melt the agar in a microwave oven. Cool to 45°C in a waterbath.

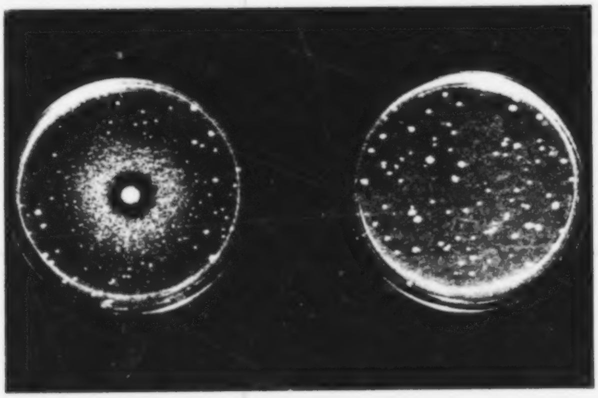

Figure 15. Spot test, Test Strain TA100 (*left*–sensi disc contains 100μl of methyl methanesulfonate; *right*–control showing spontaneous revertant colonies).

d. Prepare histidine/biotin solution as outlined in Section 5. Place histidine/biotin solution in 45°C waterbath to warm. Add 20 ml histidine/biotin solution/200 ml top agar.

Perform the following steps under a laminar-flow hood; wear fully fastened laboratory coat and surgical gloves.

e. Add appropriate concentrations of test material in duplicate to sterile 13 x 100 ml test tubes which have been placed in an ice bath (see Plate Incorporation Procedure-Primary Screen, 7.4.1, Step 'h').

f. Inoculate each tube with 0.1 ml overnight broth culture of the desired test strain.

g. Add 0.5 ml of RLE mix to each tube containing sample extract and culture.

h. Quickly mix the contents of each tube and incubate with constant agitation at 37°C for 15 min.

i. Add 2 ml of molten top agar (from Step 'd') to each tube, and rotate each tube rapidly between the palms to thoroughly mix the materials. A Vortex-mixer may be used if mixing is performed gently to prevent bubble formation in the top agar.

j. Pour the contents of each tube onto the surface of a minimal agar plate. Gently tilt and rotate each plate to spread the top agar uniformly over the surface of the minimal agar.

k. Set each plate, with lid slightly ajar, on a flat surface to dry for several minutes. Cover the plates, seal in plastic bags, and incubate at 37°C for 48 hr in an inverted position. Count the number of revertant colonies.

l. If increased numbers of mutations are noted, repeat the above steps in duplicate (preferably in triplicate) with all active strains using additional concentrations of test materials (see Plate Incorporation Procedure-Dose Response, 7.4.2).

m. Perform negative controls for each strain using phosphate buffer instead of RLE mix.

n. Perform positive controls using an appropriate diagnostic mutagen.

o. Perform control plates on solvent (DMSO) at similar concentrations.

8. QUALITY CONTROL PROCEDURES

Perform quality control tests and record results [Figures 16, 17, 18, and 19] for all mutagenicity analyses. Include analyses of distilled water extracts, analyses of test strain function and viability, sterility tests of solvents (DMSO), extracts, reagents, culture media, and exposure of test strains to diagnostic mutagens.

8.1 VIABILITY COUNTS

Bacteria density inoculated per plate is critical in determination of typical dose-responses and general reproducibility of results. Bacteria densities of the 16 hr broth cultures should be approximately 10^9 viable organisms per ml for each test strain. Routine viability counts (serial dilutions of nutrient agar pour plates) should be performed prior to analyses until an acceptable and reproducible density has been established. It may be necessary to reduce or increase the

Figure 16

QUALITY CONTROL DATA FORM-EQUIPMENT

Year: 19 ______

Month ______

Day:												
STILL												
Conductance												
pH												
Drained												
WATERBATHS												
1)												
2)												
3)												
4)												
5)												
AUTOCLAVE												
Pressure												
Temp. (max)												
OVEN												
Temp. (max)												
INCUBATORS												
1)												
2)												
3)												
4)												
5)												
REFRIGERATORS												
1)												
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FREEZERS												
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Figure 17

QUALITY CONTROL DATA FORM-MEDIA

MEDIA AND LOT #	RECEIVE	OPEN	AMT	pH	DATE	INT	MEDIA AND LOT #	RECEIVE	OPEN	AMT	pH	DATE	INT

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Figure 18

QUALITY CONTROL DATA FORM-REAGENTS

(Sterility, pH, etc.)

REAGENT	TEST	RESULT	DATE	INT	REAGENT	TEST	RESULT	DATE	INT

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Figure 19

QUALITY CONTROL DATA FORM-SPONTANEOUS REVERTANTS AND DIAGNOSTIC MUTAGENS

DATE, INITIALS MEDIA LOT #	NOTES	TA 1535	TA 1537	TA 1538	TA 98	TA 100
Spont. Revert.						
Date:	Average					
Hist/Biotin						
Biotin						
U.V. Sens.						
Amp Sens.						
C.V. Sens.						
Spont. Revert.						
Date:	Average					
Hist/Biotin						
Biotin						
U.V. Sens.						
Amp Sens.						
C.V. Sens.						
Spont. Revert.						
Date:	Average					
Hist/Biotin						
Biotin						
U.V. Sens.						
Amp Sens.						
C.V. Sens.						
	Average					
Hist/Biotin						
Biotin						
U.V. Sens.						
Amp Sens.						
C.V. Sens.						
	Average					
Hist/Biotin						
Biotin						
U.V. Sens.						
Amp Sens.						
C.V. Sens.						
MNNG						
MMS						
9AA						
4NNO						
Daun.						
2AF						

incubation period accordingly to obtain 10^9 cells per ml. After a proper incubation period has been determined, viability counts should be performed at routine intervals. As an alternate approach, correlate viability counts with spectrophotometric data so that bacteria densities can be determined quickly.

8.2 AUTOMATED COLONY COUNTS

Observed differences often occur between automated and manual counting procedures for a specific group of plates. Differences are due to several factors including: (1) masking of colonies around the periphery of the agar plates, (2) overlapping or clustered colonies which are not fully counted, and (3) bubble formation in the agar media.

Automated colony counter manufacturers supply calibration procedures which are unique to each instrument. Establish a calibration technique for each counting application (different batches of agar media, test strains, and each counting run) to verify automated with manual counts. Analysts should be thoroughly familiar with instrument operation and calibration. Examine all agar plates before use and discard those containing excessive bubbles.

8.3 DIAGNOSTIC MUTAGENS

Positive diagnostic mutagens are included with each test to confirm proper test strain function. Because it is undesirable to handle and store the concentrated mutagenic materials in a laboratory that is not equipped with elaborate safety controls (high hazard area), it is recommended that diagnostic mutagens be purchased in diluted quantities from a commercial laboratory*. Some mutagenic materials are extremely unstable. However, those dissolved in DMSO seem to retain their potency for longer periods. Routinely, check for potency of diagnostic mutagens; many are usable for 6 months or more. Dose response curves should be established for each diagnostic mutagen. This curve is used to monitor and possibly adjust the mutagen concentration over time.

---

* Diluted diagnostic mutagen standards may be obtained from Nanogens International, P. O. Box 1025, Watsonville, California 95076.

The following table contains the characteristic reversion patterns of the five standard test strains to several diagnostic mutagens. Additional mutagenic compounds may be incorporated for quality control. These include sodium azide, benzo (a) pyrene and 7,12-dimethylbenzanthracene (DMBA). Each laboratory should use positive controls at concentrations yielding reproducible counts to confirm the reversion properties of each strain. Often the choice of controls may be influenced by the suspected chemical characteristics of the sample.

CHARACTERISTIC REACTIONS OF FIVE STANDARD
SALMONELLA TEST STRAINS TO DIAGNOSTIC MUTAGENS[a]

Mutagen[b]	Amount Used (μl)	S-9 Mix	Test Strains TA 1535	TA 1537	TA 1538	TA 98	TA 100
			Mutagenic Response				
Methyl methanesulfonate	10		±[c]	-[c]	-	±	+[c]
4-Nitroquinoline-N-oxide	2.5		±	-	±	+	+
MNNG[d]	5		+	±	±	-	+
9-Aminoacridine	100		-	+	-	-	-
Daunomycin Hydrochloride	100		-	-	-	+	-
2-Aminofluorene	50	0.5 ml	-	-	+	+	+
Aflatoxin B_1	50	0.5 ml	-	-	±	+	+

[a] Plate incorporation assay.

[b] All chemicals are dissolved (1 mg/ml) in DMSO except for Daunomycin, which is dissolved (1 mg/ml) in distilled water.

[c] '+' = at least 2.5 times spontaneous reversion rate; '-' = $\leq$ spontaneous reversion rate; '±' = slightly elevated reversion rate, but less than 2.5 times spontaneous reversion rate.

[d] N-methyl-N'-nitro-N-nitrosoguanidine.

8.4 DISTILLED WATER BLANK

Fill a clean, gallon, amber-glass bottle with sterile distilled water and treat as a sample. This provides background information on the quality of the sample bottles, distilled water, extract solvents, emulsion removal and the concentration process.

Test a DMSO blank at concentrations similar to those used in the assay to ensure that this solvent is not toxic and does not interfere with test results.

8.5 STERILITY CONTROLS

Perform sterility controls on solvents, extracts, liver homogenate, RLE mix, and culture media. Examine test reagents for sterility by a "spread plate" technique. Inoculate 1 ml aliquots of test reagents onto sterile nutrient agar plates, spreading the test materials uniformly by gently tilting and rotating the plates, or spread the material with a small, sterile, glass "hockey stick".

Incubate plates at 37°C for 24 hr. Discard contaminated reagents or, if appropriate, re-sterilize. Pre-incubate all culture media at 37°C for 24 hr.

DMSO extracts are usually sterile as a result of the bactericidal action of the solvents in the extraction procedure. Occasionally, a waste concentrate may consist of viscous materials, and the extracts remain contaminated. Contaminated DMSO extracts may be filter-sterilized through a fritted-glass filter or a 0.45 μm teflon filter prior to assay.

For a more complete discussion of quality control procedures, see Microbiological Methods for Monitoring the Environment Water and Wastes[2].

9. INTERPRETING AND REPORTING RESULTS

A typical dose-response curve showing mutagenicty of a textile waste sample is presented in Figure 20. Generally, dose-response curves for pure mutagenic compounds are linear. However, non-linear curves are often observed in the case of complex materials. Misinterpretation of the mutagenic activity can result if too high concentrations of mutagenic materials are tested. High concentrations of bactericidal materials can cause excessive mortality, which results in a decrease in the number of revertants. Initial screening of suspected mutagens should include a wide range of concentrations. All positive results must be confirmed using a narrower range of concentrations. At least 500 µl of material should be tested before a negative result is reported. An example of a data summary report is shown on page 48.

Most investigators consider a chemical to be mutagenic if the number of induced revertants is two or more times greater than the number of spontaneous revertants[1]. A two-fold increase in induced revertants coincident with a typical dose-response correlates closely ($\geq$90% probability) with inducement of tumors by organic compounds in laboratory animals [1,5,6,7].

The NEIC uses a mutagenic activity ratio* (MAR) of 2.5 or greater as one of the two criteria for determination of a positive mutagenic response. Commoner and co-workers[9-11] have provided a basis for selection of this ratio. In tests of approximately 100 compounds (50 carcinogens and 50 noncarcinogens), these investigators concluded that approximately 83% of the presumptive carcinogens yield ratios above 2.0, and the same percentage of noncarcinogens yield ratios below that value. However, an MAR of 2.5 or greater increases the probability to 95%, that a substance would produce tumors if administered to laboratory animals, i.e, that it is a presumptive carcinogen.

---

* This is defined as $E-C/\bar{c}$ where E is the number of mutant colonies/plate when the compound is present; C is the corresponding value for the control, with the compound absent, obtained on the same day; and $\bar{c}$ is the average historical control value determined for each laboratory.

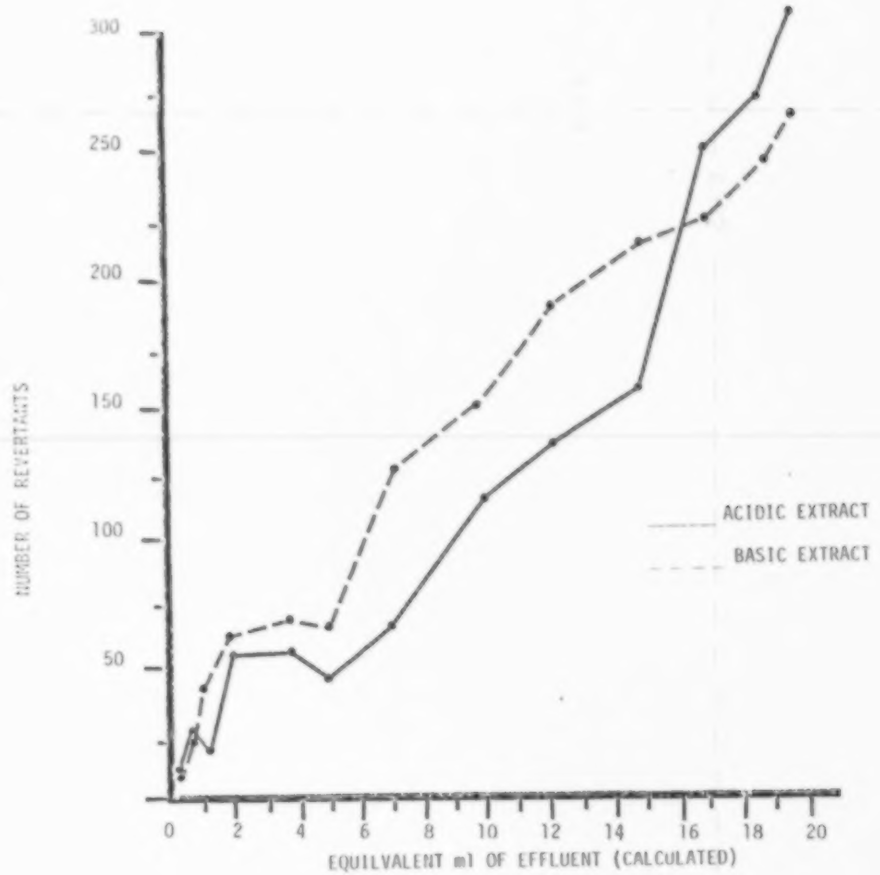

Figure 20
Dose Response Curve for Salmonella Test Strain TA 1537
Textile Plant Effluent

DATA SUMMARY, MUTAGENIC ACTIVITY OF TEXTILE MANUFACTURING COMPANY EFFLUENT
October-December 1978

Station No. & description	Sample[a] Type	Date/Time Collected	Extract pH	Salmonella Test Strain	Volume of Concentrate Tested (µl)	Equivalent Volume of Sample (ml) (Calculated)	No. of Revertant Colonies/Plate Control[b]	No. of Revertant Colonies/Plate Experimental[c]	Mutagenic Activity Ratio[d]
01	Composite	10/16/78 0600	Basic	TA 1537	100	19.4	8	264	36.5
					95	18.5		249	34.4
					85	16.5		225	31.0
					75	14.6		214	29.4
					60	11.7		181	24.7
					50	9.7		147	19.8
					35	6.8		125	16.7
					25	4.9		68	8.6
					20	3.9		69	8.7
					10	1.9		61	7.6
					5	1.0		46	5.4
					2.5	0.5		25	2.4
					1.0	0.2		9	1.0
Textile Manufacturing Co. Treatment Pond Effluent			Acidic	TA 1537	100	19.4	8	307	42.7
					95	18.5		277	38.4
					85	16.5		258	35.7
					75	14.6		166	22.6
					60	11.7		138	18.6
					50	9.7		113	15.0
					35	6.8		72	9.1
					25	4.9		48	5.7
					20	3.9		55	6.7
					10	1.9		54	6.6
					5	1.0		24	2.3
					2.5	0.5		20	2.0
					1.0	0.2		11	0.5

a Composite sample - Compositing was hourly over a 24-hr period; date and time listed is date and time period ended.
b Value based on average of two control values.
c Average of two plates
d Mutagenic Activity Ratio = $E-\underline{C}/c$ where: E is the number of colonies/experimental plate
$\underline{C}$ is the Average Control Value based on two plates
c is the historical control value averaged over 100 tests (TA 1537 = 7)

A substance is determined mutagenic if: (1) an MAR of 2.5 is obtained, and (2) a typical and reproducible dose-response is observed.

A continuing average of all spontaneous revertant controls for each test strain should be recorded. Use of this average in calculating the MAR serves to "smooth out" bias that can occur with various daily control values. With complex environmental mixtures, it is important to record the total volume of sample extracted. This measurement can be used to calculate the equivalent volume of orginal sample that resulted in mutagenic activity. A suggested data reporting form is presented in Figure 21.

Because of the limitations of the sample concentration procedure and the inherent limitations of the Salmonella mutagenicity test, a negative result from any sample is inconclusive. A negative result does not necessarily mean the sample is non-mutagenic, but that the mutagenic effect is not detectable under the conditions of the test system used. The *Salmonella* mutagenicty test measures mutations at specific base sequences in bacterial DNA; it does not indicate overall mutagenic potential.

The method described will yield a low estimate of mutagenic activity from complex materials for the following reasons: (1) the volatile alkyl halides are lost in the dichloromethane/acetone/DMSO exchange, and (2) some of the important chlorinated carcinogens such as carbon tetrachloride, chloroform, and hexachlorobenzene are not detected. Therefore, an analysis by gas chromatography/mass spectrometry should always be used to complement the *Salmonella* mutagenesis assay. Volatile organic chemical analyses should be performed, also.

10. SAFETY CONSIDERATIONS

Any laboratory equipped to do general microbiological analyses can be adapted to provide the necessary safety equipment for mutagen analyses. Simple application and adherence to "common sense" rules when handling mutagenic and carcinogenic material are extremely important.

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Figure 21

AMES ASSAY DATA FORM

DATE ________

STATION NO ________ SEQUENCE NO ________ COMPOUND NAME ________ ANALYST ________

Extract pH	Quant (ul)	S-9 Mix	Number Of Revertants					Extract pH	Quant (ul)	S-9 Mix	Number Of Revertants				
			1535	1537	1538	98	100				1535	1537	1538	98	100

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10.1 SAFETY PROCEDURES

Listed below are safety procedures and adaptations followed by the NEIC laboratory during performance of microbial mutagen analyses. The reader is referred to the EPA Safe Practices Handbook on Carcinogens[12] for additional information.

a. Perform all organic solvent extraction procedures in a properly ventilated explosion-proof hood.

b. Wear fully fastened laboratory coats and latex surgical gloves when handling mutagens or potentially mutagenic material.

c. Mouth pipetting is strictly prohibited. Perform all pipetting operations with a mechanical device so that no possible contact can be made between the laboratory workers and test materials.

d. The Federal Occupational Safety and Health Act of 1972 requires biological hazard signs and tags to identify presence of potential health hazard. Place signs properly to identify equipment, containers, rooms, materials, experimental animals, or combinations of each of these items. Where chemical carcinogens are present, post work and storage areas with signs bearing the legend: DANGER-CHEMICAL CARCINOGEN - AUTHORIZED PERSONNEL ONLY.

e. Add test materials (suspected mutagens) inside a total-exhaust laminar-flow hood to prevent worker exposure and contamination of culture materials. Each laboratory-type hood or device used for containment of mutagenic or carcinogenic materials must bear the legend: DANGER - CHEMICAL CARCINOGEN.

f. Prevent exposure of potentially toxic, volatile materials to the laboratory environment or between individual inoculated plates by sealing plates with sealable plastic bags immediately after inoculation. Open plates and plastic bags only under a properly vented laboratory hood.

g. Permit only authorized personnel to enter work and storage areas where chemical carcinogens are present.

h. Cover all work surfaces (bench tops, hood floors, etc.) on which chemical mutagens and carcinogens are used with stainless steel or plastic trays, dry absorbent plastic-backed paper, or other impervious material.

i. Keep only minimum working quantities of chemical carcinogens and mutagens in the work area.

j. Properly label storage vessels containing chemical mutagens or carcinogens. Catalog and store stock quantities of chemical mutagens and carcinogens in a specific storage area that is secured at all times.

k. Use unbreakable outer containers, such as metal desiccators to transport chemical carcinogens and mutagens.

l. Place materials contaminated with chemical carcinogens in separate sealed plastic bags or other suitable impermeable containers before transferring from work areas to disposal areas. Label both with the name of the carcinogen and a sign DANGER - CHEMICAL CARCINOGEN, before transporting.

m. Use housekeeping procedures which suppress the formation of aerosols; dry sweeping and dry mopping are prohibited.

n. Use special precautions when handling DMSO. This chemical is readily absorbed through the skin, along with any materials contained within the solvent. DMSO must be stored in a locked cabinet.

o. Autoclaving any mutagenic test materials or cultures is strictly prohibited.

p. A thorough safety procedure for handling hazardous materials, including a health monitoring program for employees, shall be established for each Laboratory.

10.2 WASTE DISPOSAL

The NEIC laboratory performs all mutagen tests using disposable test tubes, pipettes and petri dishes. All potentially toxic materials are put into plastic bags, labeled, sealed and disposed of in 55-gal plastic-lined drums. Ultimate disposal of all materials is by a private contract firm that uses an approved hazardous waste disposal site. High temperature incineration is the preferable means of disposal if this service is available.

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APPENDIX T

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Research Triangle Park, NC 27709

Submitter: Dr. Bruce C. Casto
Director of Research
Health Effects
(919)549-0611

Date of submission: January 20, 1981

Omissions: *E. coli* Assay
S. cerevisiae Assay

NORTHROP SERVICES, INC. A Subsidiary of Northrop Corporation

ENVIRONMENTAL SCIENCES

P.O. Box 12313
Research Triangle Park, N. C. 27709
Telephone: (919) 549-0611

January 20, 1981

Stephanie Toney
Genetic Bioassay Branch (MD 68)
Environmental Protection Agency
HERL, GTD, GBB
Research Triangle Park, NC 27711

Dear Ms. Toney:

Enclosed please find our Research Plan for the Development and application of microbial mutagenicity as a bioanalytical tool. If there are any specific questions you need answered concerning this please forward them to Ms. Linda Snow.

Sincerely yours,

Bruce C. Casto

Bruce C. Casto, Sc.D.
Director of Research
Health Effects

Encl.

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Environmental Protection Agency
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Research Triangle Park, NC 27711

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Sincerely yours,

Bruce C. Casto

Bruce C. Casto, Sc.D.
Director of Research
Health Effects

Encl.

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RESEARCH PLAN
TASK NUMBER
9026

DEVELOPMENT AND APPLICATION OF MICROBIAL MUTAGENICITY AS A BIOANALYTICAL TOOL

by

Linda K. Snow

Northrop Services, Inc.
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RESEARCH PLAN

Task Number 9026

Development and application of microbial mutagenicity as a bioanalytical tool

1.0 INTRODUCTION

The development and evaluation of short-term tests for the identification of carcinogens and mutagens are urgently needed if estimates of 70 - 80% for environmentally induced cancer are valid. Several methods for *in vitro* testing have been described and many new systems are being developed. It is anticipated that a battery of tests will be available with a high degree of reproducibility and correlation with *in vivo* activity. These tests should generate no false-negatives and preferably only a low percentage of false-positives, be responsive to the various classes of chemical and physical agents, and lend themselves to quantitation so that effective judgements can be made concerning the relative risk of the test agent.

Among the various short-term tests proposed for screening environmental agents for potential carcinogenicity/mutagenicity, microbial assays offer several advantages: (a) they are rapid and relatively inexpensive, (b) the assays are amenable to testing various classes of compounds through slight modifications of existing techniques, (c) they can be used to prioritize fractions from complex mixtures for further examination in animal bioassays, (d) they allow testing of agents available in only limited amounts, (e) large numbers of compounds can be tested in a given period, (f) the target molecule for mutagens is the same (DNA) for both eukaryotic or prokaryotic cells and (g) the test is relatively easy to perform and is reproducible.

Some of the possible disadvantages are: (a) some classes of chemicals are difficult to test including chlorinated hydrocarbons, metals, particulates, hydrazines and hormones, (b) the tests may be less sensitive for certain promutagens/procarcinogens than alternate mammalian cell systems, (c) the dependency upon an exogenous activating system limits the quantitative aspect of the tests and makes potency ranking of questionable value, (d) although DNA is the target molecule in both mammalian and bacterial cells for certain mutagens, differences do exist in the DNA's (replication time, available insult sites, level of DNA) and in repair mechanisms and (e) to utilize these tests most effectively will require many different strains of microorganisms together with homogenates from several different strains and organs of animals supplying the activating systems.

Even with the limitations discussed above, the Salmonella/microsome mutagenicity test has been reported to have a predictive value for carcinogens of 90% and for non-carcinogens, 87% (McCann and Ames,1977), can be used for potency rankings with certain classes of compounds, and at present provides a rapid system for screening large numbers of chemicals. Microbial tests, used in conjunction with other short-term *in vitro* tests such as mammalian cell mutagenesis and carcinogenesis assays, provide an effective alternative to animal bioassays for screening of environmental mutagens/carcinogens.

2.0 OBJECTIVES

Microbial mutagenicity bioassays will be conducted on samples from diesel and gasoline automobiles as well as important comparative source samples. The samples will consist of complex mixtures, fractions and pure compounds identified in characterization studies. Other samples to be tested will include those in support of in-house programs.

3.0 APPROACH TO OBJECTIVES

3.1 *Tester strains.* The Salmonella/Mammalian - Microsome Mutagenicity Test (Ames *et al.*, 1975) is a reverse mutation system, involving the use of five *Salmonella typhimurium* strains (TA1535, TA100, TA1537, TA1538, and TA98) which were selected by Ames for their sensitivity for reversion from a histidine requirment back to prototrophy by a wide variety of mutagens. The five tester strains possess different types of histidine mutations: strains TA1535 and TA100 possess a point mutation in the *his* G locus; TA1537, in *his* C; and TA1538 and TA98, in *his* D. In addition to the histidine mutation, each tester strain also contains two additional mutations that increase sensitivity to mutagens; a deletion of the *uvr* B gene, causing loss of excision repair, and an *rfa* (deep rough) mutation which causes loss of the lipopolysaccharide layer coating the bacterial surface. Loss of excision repair makes all five tester strains much more sensitive to any mutagen, altering DNA in a way that would normally be repaired by this system. (The *uvr* deletion also includes the biotin (bio) gene, causing all five tester strains to require biotin in addition to histidine.) The lipopolysaccharide (LPS) layer normally present on the surface of *S. typhimurium* acts as a barrier to the passage of some mutagens through the membrane; removal of the LPS by the *rfa* mutation allows mutagens of high molecular weight to enter the bacterial cells.

Two of the tester strains, TA100 and TA98, also contain R factor plasmids for ampicillin resistance. These two strains were developed by transferring plasmids into strains TA1535 and TA1538 respectively. The presence of the plasmids in TA100 and TA98 results in these strains being very effective for detecting classes of carcinogens that previously were not detected or detected only weakly using the original tester strains. The exact mechanism whereby the R factor enhances mutagenesis is not fully understood, but error-prone recombinational repair may be involved.

The five *Salmonella* tester strains, used as a set, are capable of detecting mutagens causing both frameshift mutations and base-pair substitutions. Strains TA1535 and TA100 can detect mutagens which cause base-pair substitutions. The other three strains (TA1538, TA1538 and TA98) can detect various kinds of frameshift mutagens. The five tester strains were obtained from Dr. Bruce Ames at the University of California in Berkeley and were checked out upon arrival as recommended by Ames. The check-out procedure is outlined in detail in Section 5.1.

3.2 *Storage*. Frozen permanent stocks of all the tester stains will be prepared by placing 0.8 ml of a fresh nutrient broth culture and 0.07 ml of dimethylsulfoxide in 2 ml (0.5 dram) sterile glass screw-capped vials. These culture vials will be stored in a Revco freezer at -70 C. Duplicate frozen cultures will be prepared for each tester strain so that one vial of each strain can be stored as an unopened master copy and the other used to routinely prepare master plates as described below.

3.3 *Maintenance*. The surface of an unthawed frozen culture of the bacterial strain will be scraped with a sterile wooden applicator stick. The stick will then be used to inoculate 10 ml of nutrient broth in a 125 ml Bellco Delong flask. After shaking overnight at 37 C, the nutrient broth cultures will be checked by the procedure described in Section 5.1. If the cultures check out, they will be used to prepare master plates which can be used as a source of inoculum for fresh nutrient broth cultures for mutagenesis testing.

Master plates will be Vogel Bonner Medium E plates (described in Section 3.5). In the case of master plates for TA1535, TA1537, and TA1538, 0.1 ml of 0.1 *M* L-histidine will be spread on the surfaces of the minimal media plates. In the case of master plates for TA100 and TA98, 0.1 ml of an 8mg/ml ampicillin solution will also be added, in addition to the histidine, as a selection pressure for retaining the plasmids.

Master plates will be used as a source of inoculum for nutrient broth cultures for no longer than 2 months, at which time new master plates will be prepared from frozen permanents and the strains checked out as previously described.

Nutrient broth overnight cultures of the tester strains will be grown up fresh for each experiment using a single colony from each master plate as inoculum.

3.4 *Rat liver S-9 Fraction and S-9 Mix.* Ultimate carcinogens are often directly mutagenic, while procarcinogens require metabolic activation in order to manifest their mutagenic activity. Metabolic activation can be accomplished in bacterial testing systems by the addition of rat liver homogenates (S-9 fraction) directly to Petri plates, thus incorporating an aspect of mammalian metabolism directly into an *in vitro* test.

Induced S-9 fraction will be obtained from young male rats, Charles River strain CDI, weighing 100-150 g. The inducer used will be Aroclor 1254. The option of also using non-induced S-9 fraction will be maintained.

The induction procedure and the procedure for obtaining the liver fractions are described in the Biochemistry work plan for Technical Directive 4.7-1.

The fresh S-9 fractions will be frozen in liquid nitrogen and stored in a Revco freezer at -70 C. As required, sufficient S-9 fraction will be thawed overnight in the refrigerator with the unused portion being discarded at the end of the day.

The microsomal enzymes in the S-9 fraction capable of activating procarcinogens (i.e. mixed function oxidases or aryl hydrocarbon hydroxylase) require NADPH for activity. This required NADPH is generated in what Ames refers to as S-9 mix.

S-9 mix will contain per ml:

S-9 fraction from rat livers (0.1 ml)
magnesium chloride (8 μmoles)
potassium chloride (33 μmoles)
glucose-6-phosphate (5 μmoles)
NADP (4 μmoles)
sodium phosphate buffer, pH 7.4 (100 μmoles)

Stock solutions of NADP (0.1 *M*) and glucose-6-phosphate (1 *M*) are stored frozen at - 20 C. The stock salt solution (0.4 *M* magnesium chloride/1.65 *M* potassium chloride) and phosphate buffer (0.2 *M*, pH 7.4) are refrigerated.

3.5 *Media.* The minimal medium used will be Vogel-Bonner Medium E (VBME) containing per liter: 0.2 gm $MgSO_4 \cdot 7H_2O$, 2.0 gm citric acid monohydrate, 3.5 gm $NaNH_4HPO_4 \cdot 4\ H_2O$, 10 gm K_2HPO_4, 15 gm Difco agar, and 20 gm glucose. After autoclaving, 2.5 ml of a sterile solution of histidine and biotin (210 mg L-histidine/390. 4 mg d-biotin/liter) will be added to each liter of medium. This solution provides excess biotin (29.28 μg per plate) and a trace amount of histidine (15.75 μg per plate) to enable the bacteria to undergo several divisions; this growth is necessary in many cases for mutation to occur.

All plates will be prepared automatically using a plate-making machine. Plates will be 100 x 15 mm, and each plate will receive 30 ml of agar. After preparation, plates will be incubated overnight at 37 C and then stored in the refrigerator in plastic bags until used.

Histidine$^-$(his$^-$) top agar will contain 6 gm agar and 5 gm of sodium chloride per liter of distilled water. Histidine $^+$(his$^+$) top agar will contain 6 gm agar, 5 gm sodium chloride and 39 mg of L-Histidine per liter of distilled water. NADPH solution will contain 0.032 gm NADPH per 10 ml of sterile distilled water.

4.0 PROTOCOLS

4.1 *Assays for mutagenicity of selected chemicals.*

4.1.1 *Plate incorporation assay.* Top agar will be dispensed into small test tubes in 2 ml quantities. The following will then be adden (in order) to each 2 ml quantity of molten (his$^-$) top agar at 46C: 0.1 ml of an overnight nutrient broth culture (approx. 2 x 10^8 cells) of the bacterial tester strain, the various amounts of sample or control mutagen to be tested and 0.5 ml of the S-9 mix (where appropriate). The contents will be stirred on a Vortex mixer and poured onto the VBME minimal media plates previously described; plates will then be placed in a dark, 37 C incubator. After 48 hr,revertants appearing on the plates will be counted using a Biotran III automatic colony counter. Plates negative at 48 hr will be incubated for an additional 24 hr before discarding.

4.1.2 *Spot tests.* The methods for spot testing of samples are the same as the plate incorporation assay except that the sample or control mutagen is omitted from the top agar, but is applied to the agar surface after the top agar containing the bacteria and S-9 mix is poured. The sample can be added to the agar surface as crystals, as a micro drop, or on a sterile filter paper disc.

4.1.3 *Suspension assay.* Nutrient broth overnight cultures of each tester strain will be centrifuged, resuspended in saline, and adjusted to a concentration of 1 x 10^9 bacteria/ml. The following will then be added to each test flask: 0.9 ml sodium phosphate buffer pH 7.4, 1.0 ml of bacteria,1.0 ml of NADPH solution, 0.1 ml of the sample or control mutagen to be tested, 1.0 ml of S-9 fraction (when appropriate) and sterile water to bring the total volume in each flask to 4.0 mls.

All test flasks will then be incubated in a 37 C shaker for 60 min unless otherwise requested. At the end of the incubation period, a 10^{-6} saline dilution of an aliquot from each flask will be prepared. Both reversion and survival will be measured. Reversion will be measured by adding 0.5 ml of undiluted flask contents to 2 ml of melted his^- top agar and then pouring the agar onto a VBME plate. Survival will be measured by adding 0.5 ml of the 10^{-6} dilution of the flask contents to 2 mls of melted his^+ top agar and then pouring the contents onto a VBME plate. Incubation and counting of plates will be as described as for the plate incorporation assay.

4.2 *Conduct of test.*

4.2.1 *Routine testing.* Routine diesel exhaust samples will be dissolved in dimethylsulfoxide and tested by plate incorporation. Tester strains and sample doses employed will be speicfied by EPA technical monitors. Each sample will be evaluated in the presence and absence of liver S-9 activation unless otherwise requested.

4.2.2 *Microsome testing.* Preliminary preparation will consist of: (a) dilution of each sterile microsome sample to obtain four different protein concentrations (e.g. 0.3 mg, 0.1 mg, 0.03 mg, and 0.01 mg), (b) preparation of a cofactor mix referred to a "NADP mix". This mix will be prepared such that each plate will receive 0.75 mM NADP,10 mM G-6-P, and 2 units of G-6-PDH.

The enzymatic activity of each sample concentration will then be determined by a modified plate incorporation using two standard mutagens as indicators. Amounts to be added (in order) to each his^- 2 ml top agar tube will be : 0.1 ml of an overnight nutrient broth culture of the specified bacterial tester strain, 0.1 ml of the appropriate mutagen dilution, 0.1 ml of NADP mix, and the appropriate amount of sample protein. When AAF is employed as a standard mutagen in microsome testing, 0.1 ml of 100,000 x gm supernatant will also be added to each agar tube.

4.2.3 *Urine testing.* Preliminary preparation will consist of: (a) filter sterilization of the urine samples to be tested. (b) preparation of a sterile 7,000 unit/ml solution of B-glucuronidase.

The mutagenic activity of each urine sample will then be determined by a modified plate incorporation assay. Amounts to be added (in order) to each 2 ml his^- top agar will be: 0.1 ml of an overnight nutrient broth culture of the bacterial tester strain, a specified dose of the urine sample, 0.1 ml of the B-glucuronidase solution (700 units) and 0.25 ml of S-9 mix. (The total S-9 mix will contain 0.3 ml S-9 fraction per ml rather than the standard 0.1 ml of S-9 per ml of mix).

Tester strains and sample doses employed will be specified by EPA technical monitors. Each sample will be evaluated in the presence and absence of liver S-9 activation unless otherwise requested.

5.0 QUALITY ASSURANCE

5.1 *Checking of tester strains.* Checking of the five tester strains will be performed on a routine basis. It should be noted that this is essential, especially in the case of the unstable plasmid containing strains TA100 and TA98. The properties of the tester strains will be evaluated each time the strains are cultured from frozen permanent stocks and will consist of five parts as described below:

5.1.1 *Histidine requirement.* Nutrient broth cultures will be streaked on minimal media plates both with and without histidine (0.1 ml of 0.1 *M* L-histidine spread on agar surface). After 48 hrs at 37 C, growth should be observed on all plates spread with histidine. No growth should occur on plates without histidine.

5.1.2 *Presence of deep rough (rfa) character.* Nutrient broth cultures will be tested for crystal violet sensitivity. A sterile filter paper disc containing crystal violet (approximately 0.1 ml of a 1 mg/ml solution) will be placed on a nutrient agar Petri dish containing 0.1 ml of the culture in 2 ml of top agar overlay. After 24 hrs at 37 C, a clear zone of growth inhibition should be present around the disc.

5.1.3 *Presence of the R factor plasmids.* Nutrient broth cultures will be tested for ampicillin sensitivity. A sterile filter paper disc containing ampicillin (50 µl of a 8 mg/ml solution in 0.02 *N* NaOH) will be placed on a nutrient agar Petri dish containing 0.1 ml of the culture in 2 ml of top agar overlay. After

24 hrs at 37 C, strains which do not contain the R factor should show a zone of inhibition around the disc, whereas R factor containing strains should not.

5.1.4 *Presence of uvr B deletion.* Nutrient broth cultures will be tested for sensitivity to ultraviolet light. 0.1 ml of each culture will be placed in 2 ml of top agar and poured onto a nutrient agar plate. Half of each plate will then be irradiated with UV (ultraviolet light) for 25 seconds under a GE G64T6 lamp at a distance of 66 cm (surface intensity approximately 200 $\mu W/cm^2$). After 24 hrs at 37 C, growth should occur only on the unirradiated side of the plate.

5.1.5 *Spontaneous reversion rate.* The spontaneous reversion will be checked for each strain and a time course record of the results will be kept.

5.2 *Controls.* Quality control will be carefully maintained. Positive control mutagens, those requiring and those not requiring S-9 activation, will be used in each experiment to verify the responsiveness of the tester strains and the activity of the S-9 mix.

5.2.1 *Control mutagens.* Specific control mutagens to be routinely used for each strain are listed below:

STRAIN	CONTROL MUTAGEN	
	Nonactivation	Activation
TA1535	Na Azide	2-anthramine
TA100	Na Azide	2-anthramine
TA1537	9AA	2-anthramine
TA1538	2NF	2-anthramine
TA98	2NF	2-anthramine

Negative solvent controls, consisting of the solvent alone and the bacteria, with and without the addition of S-9 mix, will also be run to demonstrate that the solvent being used has negligible mutagenic and toxic effects on the bacteria.

5.2.2 *Sterility will also be carefully maintained.* Newly prepared solutions of samples and the S-9 mix will be routinely plated on nutrient agar to assure their sterility.

5.2.3 *All mutagenicity tests will be performed at least in duplicate.*

5.2.4 *Confirmation of mutants.* After the number of colonies on each plate is determined, representative samples of the revertant colonies will be picked from plates exhibiting a positive response. These colonies will then be replica-plated onto minimal agar to confirm if they are revertants or potential phenocopies.

5.2.5 *Toxicity.* The background lawn of bacterial growth on the test plates will be routinely examined for determination of toxic effects.

6.0 CAPABILITY AND IMPLEMENTATION OF OTHER SUB-MAMMALIAN TEST SYSTEMS.

6.1 *Capability.* Our capability is based upon the performance of the following experiments:

(a) Routine testing - *e.g.* diesel exhaust fractions or other samples not requiring prior preparation.

(b) Microsome testing - *e.g.* determination of activation capacity of rat liver preparations stimulated with various chemicals.

(c) Urine testing - *e.g.* determination of mutagenic activity of filter-sterilized, B-glucuronidase-treated urine samples.

For the basis of evaluation, one experiment will be defined as:

(a) Testing one sample at 5 doses in 5 tester strains with and without activation.

(b) Testing 5 samples at 5 doses in one tester strain.

(c) Determination of activity of four microsome preparations at four protein concentrations against two standard mutagens in one tester strain.

(d) Analysis of mutagenic activity of one sterile urine sample at 5 doses in 5 tester strains with and without activation.

Using the above categorized experiments, the laboratory personnel as presently constituted could perform six (6) complete experiments per week. This schedule would permit testing of 30 routine samples per week or approximately 1500 samples per year in one *Salmonella* tester strain.

6.2 *Development of other test systems.* Between major test sampling periods, a portion of our effort will be devoted to implementation of the following test systems.

6.2.1 *Escherichia coli DNA Polymerase Deficient (pol A$^-$) Assay.* The *Escherichia coli* DNA polymerase deficient (*pol* A$^-$) assay was originally proposed by Slater *et al*, 1971. It is based on the observation that a strain deficient in DNA polymerase (and therefore unable to repair DNA damage) is much more sensitive to agents that damage cellular DNA than the competent parent strain. A screening procedure based upon this greater sensitivity is expressed as an inhibition of growth, i.e. toxicity of the deficient strain.

Specific strains used in the procedure are *Escherichia coli* W3110 (*pol A$^+$*) and *Escherichia coli* p3478 (*pol A$^-$*).

The chief limitation of the original *pol A$^-$* assay is that it does not work well for testing substances that do not diffuse rapidly in agar. This limitation can be overcome by performing the test in liquid suspension. (Rosenkranz *et al.*1976).

It has been noted that the Ames *Salmonella* assay does not respond to some substances which give positive results in the *pol A$^-$* system. For this reason, it has been suggested that the two microbial assays should be used in tandem.

6.2.2 *Escherichia coli WP2 Mutagenicity Assay* (Green and Muriel 1976).

The *Escherichia coli* WP2 mutagenicity assay is similar to the Ames *Salmonella* assay in being a reverse mutation system; strain WP2 and its derivatives are reverted from a tryptophan requirement to prototrophy by the mutagenic action of various chemicals. In addition, agents causing many types of DNA damage can be detected through increased killing of repair deficient derivatives of WP2.

The strains suggested for routine testing are WP2, WP2 *uvr* A, WP67, and CM611. All four strains contain the tryptophan mutation. Strain WP2 *uvr* A also contains a mutation which causes loss of excision repair. Strain WP67 contains in addition to *uvr* A, a *pol* A mutation which results in the loss of the enzyme DNA polymerase I. Strain CM611 is deficient in both excision repair (*uvr* A) and error prone rec-dependent repair (*exr* A or *lex* A). The WP2 mutagenecity assay can detect mutagens which cause base repair substitutions. It cannot detect frameshift mutagens.

It has been suggested that the WP2 system is complementary to the Ames *Salmonella* assay because it is more versatile and informative for the characterization of base pair substitution mutagens.

6.2.3 *Saccharomyces cerevisiae Mitotic Recombination Assays* (Zimmerman 1971; Zimmerman 1975).

Different strains of the yeast *Saccharomyces cerevisiae* can be used to detect the induction of mitotic crossing-over and mitotic gene conversions. The strain used for studies of mitotic crossing-over is D5, a diploid strain carrying two different alleles of the gene locus *ade 2* - *ade 2-40* which causes an absolute requirement for adenine and formation of deep red colonies on low adenine medium and *ade-2-119* which causes a "leaky" requirement for adenine and formation of pink colonies on low-adenine medium. The two alleles complement each other so that D5 cells carrying both alleles form white colonies and do not require adenine at all. Induction of mitotic crossing-over in D5 cells causes the formation of colonies that have a red and pink sector twin-spotted colonies. (Cells in red spot are homoallelic *ade* 2-40; cells in pink spots are homoallelic *ade* 2-119).

Mitotic gene conversion is studied using the diploid strain D4 which is heteroallelic at two loci - *ade* 2-1/*ade* 2-2 and *trp* 5-12/*trp* 5-27. Induction of mitotic gene conversion is followed by plating D4 cells on two different selective media; tryptophan-free medium selects for convertants at the *trp* locus while adenine-free medium selects for convertants at the *ade* 2 locus. Appropriate yeast strains can also be used to detect both forward and reverse mutations.

Bacterial mutagenicity assays focus on the induction of point mutations in haploid procaryotic organisms. They cannot, however, reveal other genetic effects of mutagenic treatment such as mitotic crossing-over and mitotic gene conversion. These mitotic recombinational events can easily be studied in diploid yeast strains. The use of yeast assays in mutagenicity screening is therefore recommended as a complement to the standard bacterial assays.

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APPENDIX

SAFETY PROTOCOLS FOR TOXIC SUBSTANCES

I. PROCEDURES FOR STORAGE AND HANDLING OF CARCINOGENIC/MUTAGENIC SUBSTANCES

A. Storage

Vials and tubes containing chemical carcinogens are stored in sealed metal containers inside a locked steel solvent cabinet or freezer located in the Hazardous Materials Laboratory (HML). If a sample is suspected to have any appreciable vapor pressure, storage will be in a sealed glass vial inside the metal containers.

II. FACILITY

A. Hazardous Materials Laboratory

Located in the Northrop Environmental Technology Center, the Hazardous Materials Laboratory provides for the safe handling, storage, and analysis of known and/or potentially toxic, carcinogenic, teratogenic, and/or mutagenic chemicals and samples. The HML, maintained at progressively increasing negative pressures to prevent contamination, consists of four distinct areas.

The two laboratory work areas contain stainless steel benches, exhaust hoods, and two Class III vented gloveboxes that are equipped with air locks, HEPA and charcoal filters, and the capabilities for negative pressure and inert atmosphere operations. The Vapor Phase Preparation Lab is equipped with water baths, balances, a glovebox, and a large recording microbalance. This area is used for preparation of permeation and diffusion tubes and of air samples approximating environmental conditions and concentrations. The High Hazard Lab is used when the concentrated toxic compounds handled by the laboratory are processed. Adjoining the High Hazard Lab is the specialized hazardous materials storage facility which houses the more than 750 toxic and hazardous substances available at HML. Equipped with ventilated storage cabinets and explosion-proof refrigerator and freezer, the storage facility is wired to "vapor-proof" specifications.

The pressures, humidification, and temperatures within the HML are regulated and monitored with a centralized environmental control panel. The ventilation system ensures 22 changes of conditioned air every hour; all exhaust (including hood and glovebox exhaust) is vented through a bank of HEPA and charcoal filters. The general layout of the HML and containment facility is shown in the figure 1.

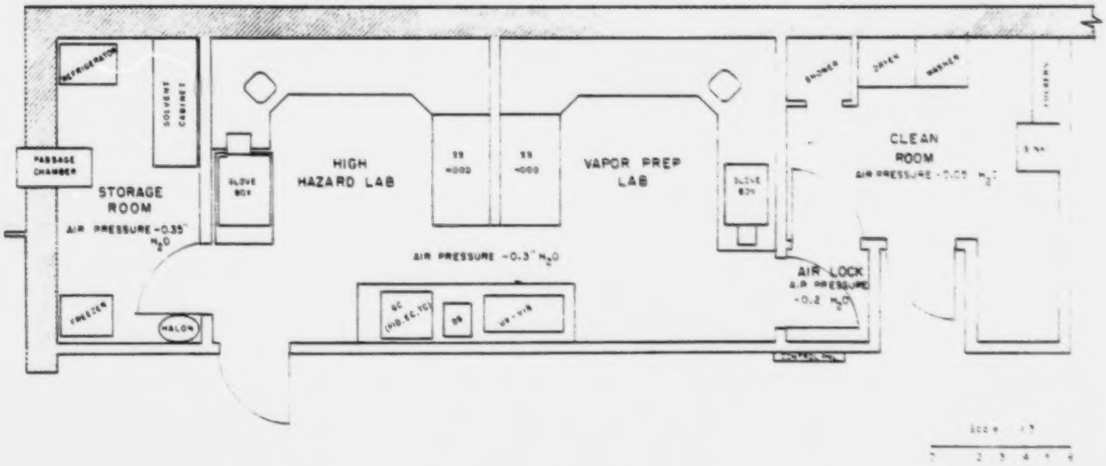

Figure 1. Hazardous Material Laboratory floor plan.

Safety is a critical concern in any operation that handles hazardous materials. NSI-ESC's Laboratory complies with all applicable OSHA and NCI regulations and safety standards. A Halon 1301 fire suppression system protects the laboratory and storage areas from organic flash fires. Strict access control, comprehensive personnel training, a complete medical surveillance program, protective equipment, and detailed protocols also work to guarantee safety to personnel and to the environment.

B. Major Analytical Equipment

Tracor 550 Gas Chromatograph equipped with glass S.C.O.T. and packed columns, automated Tenax Cartridge Desorption System, sampling valves, TC and EC detectors and FID.

Hewlett Packard 3385 Recording Gas Chromatographic Integrator system.

Hatachi 200 Recording UV-Visible spectrophotometer.

Cahn-1000 Recording Microbalance equipped with permeation tube assembly.

Cahn-RG Microbalance modified for glovebox use and ease of decontamination.

Labconco Chemical Carcinogen Gloveboxes modified for handling sensitive compounds under a nitrogen atmosphere and for ease of decontamination.

C. Staff

The staff of the HML have considerable expertise in handling preparing, and analysing hazardous substances. The following resumes summarize the professional experience of key HML personnel.

H. Erle Janssen
Supervisor, Hazardous Materials Laboratory
Toxic Substances Control Officer
MS, Environmental Engineering, Texas A&M University, 1976
BS, Environmental Sciences and Chemistry, Sam Houston State University, 1974

Mr. Janssen is the senior chemist in the NSI Hazardous Materials Laboratory support EPA-RTP. He designed and set-up the operation of this high-hazard analytical facility. Specific responsibilities included development of the protocol, safe handling procedures, inventory control, analytical procedures, and training techniques for personnel handling odorous and fluorescent tracer chemicals. He also serves as a technical advisor to both NSI and EPA scientific personnel in areas relating to laboratory safety, handling and use of toxic substances, and application of current regulations dealing with the storage and laboratory use of hazardous agents.

In a previous assignment at NSI-RTP, Mr. Janssen conducted research on pesticide residue analysis in the Sample Preparation Laboratory, Pesticide Chemistry section. He initiated and executed research projects on extraction and clean-up methodology for chlorinated hydrocarbons with heavy emphasis on gas chromatographic detection techniques. He supervised one laboratory technician and was in charge of GC operations for the laboratory.

DOUGLAS B. KIRKTON
Work Planning and Safety Coordinator
Graduate Studies in Industrial Hygiene, Air Pollution and Occupational Health, School of Public Health, UNC, Chapel Hill, 1971.
A.B. Zoology Chemistry, UNC at Chapel Hill, 1969

Mr. Kirkton is currently employed as a scientist working in the Hazardous Materials Laboratory with Northrop Services, Inc. at Research Triangle Park, N. C. He is involved in the day-to-day operations of the HML and is responsible for the identification and analysis of toxins and suspected hazardous materials. He conducts day-to-day analyses using the following types of instrumentation: capillary columns, E.C. detectors, automated chromatography systems, FID, and UV and IR Spectrophotometry. He has also been involved in the design and preparation of permeation tubes and the development of high hazard containment systems. Mr. Kirkton is knowledgeable in safety rules and regulations, OSHA Standards, and the effect of chemicals on the human system.

III. HANDLING PROCEDURES

Under no circumstance is a known or suspect carcinogenic substance opened outside of the HML prepared for handling of this material.

The container with chemical carcinogen is removed from storage and placed in a glove box within the HML. The glove box is equipped with an electronic balance and vented to the outside (HEPA and charcoal double filters) to prevent release of hazardous vapors. The glove box exhaust fan is turned on for the entrance and removal of the container. The sample is removed and a predetermined amount is weighed and added to a disposable tube or flask. The remainder is returned to the secondary sample container which is tightly capped before being placed in the tertiary container and removed from the glove box.

The carcinogenic substances will be dissolved in the proper solvent and all dilutions made in the glove box. The diluted primary material is then transferred through the pass-through taken to the appropriate laboratory, and placed in a Bio-Gard laminar flow hood; 70% recirculated air (exhaust air is HEPA and charcoal filtered).

The hood will have been prepared previously by installing new absorbent (polyethylene backing) paper and operating the hood air system for a minimum of 30 minutes prior to use. All necessary equipment, media, pipets, flasks, and a waste con-container are placed in the hood prior to the introduction of the carcinogen/mutagen. The researcher then makes all necessary dilutions and additions to cultures without removing any material from the hood. All pipetting is done with an automatic pipetter. No material inside of the hood is allowed to come in contact with any part of the researcher who is clothed in disposable gloves and gown.

After treating, the cultures containing the toxic substances are immediately removed from the hood and placed in the incubator. All cultures exposed to any carcinogenic agent are incubated in 37° incubators equipped with negative exhaust venting systems. Cultures exposed to gases are contained within a sealed modular incubator chamber prior to placing the cultures in the incubators.

IV. DISPOSAL

All solid waste is removed from the hood in a Biohazard plastic bag and transfered to appropriate waste drums. Liquid waste is added to plastic vermiculite-filled jars located within the hood. Concentrated Wescodyne is added to the jars to inhibit growth of organisms. When filled, the jars are removed and placed in a liquid waste barrel. Vermiculite is added to the drum and packed around and on top of the discarded jars.

After work in the hood is completed, all equipment which has come in contact with an unknown or known toxic substance is carefully cleaned with water followed by 70% ethanol. All equipment is removed from the hood. Decontamination of the internal surfaces is repeated after removal of equipment. The work area is checked carefully for spilled or splashed media. The cloth used to clean the hood is put into a plastic bag. The researcher then removes all protective clothing which is put into the same bag. The bag is then put into the solid waste drum.

V. MEDICAL SURVEILLANCE AND EMERGENCY PROCEDURES

No new employee will be allowed to work with the above samples until a physical examination is given to establish a baseline against which changes can be measured and to determine whether a pre-exixting condition might lead to increased risk in this work situation.

Many of the environmental samples contain known or suspected carcinogens, so care is always taken to avoid accidental exposure. In the event of accidental exposure of one of the above substances to skin or eyes, the affected area will be washed with copious quantities of water (soap and water if skin exposure). This will be followed by a call to the company physican regarding medical surveillance.

APPENDIX U

Laboratory: Raltech Scientific Services
P.O. Box 7545
Madison, WI 53707

Submitter: Dr. Robert A. Finch
Head, Genetic Toxicology
(608)241-4471

Date of submission: February 10, 1981

Omissions: None

Raltech
SCIENTIFIC SERVICES

A Division of Ralston Purina Company

February 10, 1981

Ms. Stephanie Toney
Genetic Bioassay Branch (MD68)
Health Effects Research Laboratory
U S Environmental Protection Agency
Research Triangle Park NC 27711

Dear Ms. Toney

Enclosed is a copy of Raltech's Standard Operating Procedure for the Ames Plate Incorporation Test. I am sorry for the delay, but it was necessary to obtain my management's approval to send the SOP to a non-client.

I would appreciate receiving a copy of the final compilation of protocols when it becomes available.

If I can be of any further help, please do not hesitate to contact me.

Sincerely

Bob Finch

Robert A. Finch, PhD
Head, Genetic Toxicology

RAF/cja
Enclosure

P O Box 7545 • Madison Wisconsin 53707 • 608/241-4471
Other Raltech Scientific Services Laboratories • St Louis MO • Gray Summit MO

TECHNICAL OPERATING PROCEDURE

OP-TOX 808
PAGE 1 OF 12
DATE: 10/13/80
REPLACES: 4/25/80

PROCEDURE TITLE: Plate Incorporation Method of the Ames *Salmonella*/Microsome Mutagenicity Test

AREA OF APPLICABILITY: Raltech Scientific Services - Madison
Toxicology Department
Genetic Toxicology Section

SCOPE:

This is a bacterial screening test for chemical mutagens causing gene mutations. It is applicable to pure chemicals, complex mixtures and extracts that are soluble in water or organic solvents. This test is not directly applicable to samples containing free histidine, such as natural or food products. Solvent extracts free of free histidine from natural or food products can be tested.

SAFETY:

1. Observe all standard laboratory safety procedures as described in the Raltech Scientific Services Laboratory Safety Manual, as well as those recommended in, *Guidelines for the Laboratory Use of Chemical Substances Posing a Potential Occupational Carcinogenic Risk*, Revised Draft, June 5, 1979, from the Laboratory Chemical Carcinogen Safety Standards Subcommittee of the DHEW Committee to Coordinate Toxicology and Related Programs.

2. Disposable gloves are used when handling test articles and positive controls.

3. A micropipetting system is used for all pipetting involving test articles and positive controls.

PRINCIPLE:

Five mutant strains of *Salmonella typhimurium*, TA98, TA100, TA1535, TA1537, and TA1538, which require histidine for growth, are used as the indicator cells. When exposed to mutagens, one or more of these strains will revert from a histidine-requiring auxotrophic state back to prototrophy (wild type) which do not require histidine, thus indicating the mutagenic activity of the test article. Incorporation in the test of an S-9 microsomal fraction prepared from a homogenate of Aroclor 1254-induced rat liver, permits the detection of a wide range of mutagens that require mammalian metabolic activation.

INTERFERENCES:

A test article containing free histidine can give a false positive.

A test article run at dose levels which are toxic to the tester strains can give a false-positive result.

SENSIVITY, PRECISION, ACCURACY:

Not completely established at the time of this writing.

QUALITY, ASSURANCE:

At the time the revertant colonies are counted, the background lawn of bacterial growth on each test plate should be examined microscopically and compared with the corresponding solvent control plates. If the background lawn is sparse and/or unevenly distributed on the plate, compared with the corresponding solvent control, this indicates a cytotoxic effect of the test article on the Salmonella tester strain. A cytotoxic effect of the test article can result in the growth of small non-revertant colonies which could be mistaken for revertant colonies. This could lead to a false-positive result.

Each time a new set of tester-strain Master Plates is prepared, the genetic integrity of the strains should be checked by the following methods:

1. To test for histidine and biotin requirements, streak all five tester strains on minimal glucose agar plates both with and without histidine. On the histidine plates, spread 0.1 mL of a sterile solution containing histidine (0.1 M) and biotin (0.5 mM) on the agar surface. All tester strains should grow only on the plates containing histidine and biotin.

2. To test for the presence of the deep rough (rfa) character, all the tester strains are spread on nutrient agar plates and a sterile filter paper disc containing crystal violet (10 µL of 1 mg/mL) is placed on the surface of the agar in each plate. After overnight incubation at 37°C, a clear zone of growth inhibition around each disc (∿ 14 mm diameter) should be present in all the tester strains indicating the presence of the deep rough (rfa) character.

3. To test for the presence of the R factors in tester strains TA98 and TA100, dilute the overnight culture geometrically to 10^{-5} with sterile 0.9% NaCl. Plate 0.05 mL, in triplicate, on nutrient agar plates and on nutrient agar plates to which ampicillin has been added (0.1 mL of an 8 mg/mL ampicillin solution, spread with sterile glass spreader and

allowed to diffuse overnight). Incubate the plates, inoculated with bacteria, overnight at 37°C and count the colonies. Compare the average number of colonies on the ampicillin$^+$ plates with the average on the ampicillin$^-$ plates. Ideally, they should be the same, or very close, indicating a high R-factor titer.

4. The uvr B deletion is quite stable and its presence in the tester strains is not routinely determined. Its presence can be determined using the method described by Ames _et al_ (1975).

The number of spontaneous (background) revertant colonies growing on the solvent control plates, with or without the S-9 activation system, should be within the following ranges:

TA98: 20-50
TA100: 100-200
TA1535: 10-40
TA1537: 3-25
TA1538: 10-35

APPARATUS:

1. Incubator, 37°C.
2. Quebec (Darkfield) Colony Counter (American Optical Corp.).
3. Biotran II Automated Colony Counter - Model C111 (New Brunswick Scientific Co., Inc.).
4. Water bath, 45°C.
5. Shaker water bath, 37°C.
6. Vortex mixer.
7. Standard bacteriological glassware.
8. Micropipetting system.

BACTERIA:

The five _Salmonella_ tester strains, TA98, TA100, TA1535, TA1537, and TA1538, were obtained from Dr. B. Ames in the Department of Biochemistry at the University of California-Berkeley. Reserve stocks of the tester strains are stored frozen at -90°C in nutrient broth containing 8% dimethylsulfoxide (DMSO). One set of frozen stocks is used to prepare Master Plates and the other frozen stocks are retained as Master Copies and are only used to generate cultures from which new frozen stocks for routine use are made.

REAGENTS:

Culture Media

Minimal Glucose Agar

15 g Difco Bacto-agar
1000 mL Milli-Q* deionized water.

Autoclave for 20 minutes at 15 psi (121°C). Use slow exhaust. Remove from autoclave and mix by swirling.

Add to the hot agar solution:

20 mL VB salt solution (50x concentrate)
50 mL 40% glucose solution.

Mix by swirling and pour ∿ 30 mL into each 100-mm "Muta-Assay" plates (Falcon Labware, Oxnard, California, catalog #1028). The plates are allowed to stand at room temperature until the agar has solidified and the condensation on the inside of the lids has evaporated. The plates are then returned to their plastic sleeves and stored at room temperature until used. The plates can be stored for 3-4 weeks at room temperature.

VB (Vogel-Bonner) Salt Solution (50x concentrate)

670 mL	Milli-Q deionized water (warm, 45°C)
10 g	$MgSO_4 \cdot 7H_2O$
100 g	Citric acid monohydrate
500 g	K_2HPO_4 (anhydrous)
175 g	$NaHNH_4PO_4 \cdot 4H_2O$

Add salts in the order indicated. Dissolve each salt completely before adding the next one. When all the salts are dissolved, allow solution to cool to room temperature and dispense in 20-mL aliquots into screw-cap tubes (20 x 150 mm). The tubes containing the solution are autoclaved for 15 minutes at 15 psi (121°C). Use slow exhaust. Solution is stable at room temperature for 1 year.

40% Glucose Solution

400 g glucose is brought to 1000 mL with Milli-Q deionized water.

---

* Trademark - Millipore Corporation, Bedford, Massachusetts

The glucose is dissolved completely in the water and filter sterilized by passage through a membrane filter with a 0.45 μ pore size. The solution is stored at room temperature in sterile 500-mL serum bottles and is stable for 1 year.

Top Agar

1000 mL	Milli-Q deionized water
6 g	Difco Bacto-agar
5 g	NaCl

Heat mixture to boiling in order to dissolve the agar. Take care not to scorch the undissolved agar during the heating process. After the agar is completely dissolved, cool to approximately 50°C and transfer, in 100-mL and 400-mL aliquots, to glass serum bottles. Autoclave the top agar for 15 minutes at 15 psi (121°C). Top agar is stable at room temperature for 1 year.

Before use, melt the agar by steaming the bottles in the autoclave at 100°C. After the agar is melted, add 10 mL of a solution containing 0.5 mM L-histidine HCl and 0.5 mM biotin to each 100-mL bottle of melted agar and mix by swirling.

0.5 mM Histidine - 0.5 mM Biotin Solution

Add 61 mg biotin to 10 mL pure (no additives) 95% ethanol. Add ~ 40 mL Milli-Q deionized water and autoclave for ~ 5 minutes to dissolve biotin.

Combine biotin solution with 38.75 mg L-histidine HCl and bring to 500 mL with Milli-Q deionized water. Filter sterilize by passage through a membrane filter with a 0.45 μ pore size. Stable for 1 year at 4°C.

Nutrient Broth

1000 mL	Milli-Q deionized water
25 g	Oxoid Nutrient Medium #2

Dissolve medium completely, transfer 5-mL aliquots to screw-cap tubes (20 x 150 mm) and autoclave for 15 minutes at 15 psi (121°C). Use slow exhaust. The nutrient broth is stable for 1 year at 4°C.

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Nutrient Agar

23 g	Difco Bacto Nutrient Agar
1000 mL	Milli-Q deioniized water

Autoclave for 15 minutes at 15 psi (121°C). Use slow exhaust. Remove from autoclave and mix by swirling. Pour ∿ 20 mL into each 100-mm "Muta-Assay" plates. The plates are handled the same as the minimal glucose agar plates.

Positive Control Mutagen Stock Solutions

Tester Strain	Positive Control Mutagen	ug/10 µL
TA 98	4-Nitroquinoline-N-oxide	1
TA100	Benzo(a)pyrene	5
TA1535	N-methyl-N'-nitro-N-nitrosoguanidine	4
TA1537	9-Aminoacridine	100
TA1538	2-Aminofluorene	10

The mutagens are weighed in a room illuminated with subdued light and the stock solutions are prepared in a room illuminated with yellow light. All positive mutagen solutions should be handled with a micropipetting system with polypropylene tips. Stock solutions of these mutagens are prepared in DMSO at concentrations that will give the appropriate amount of the mutagen in a 10-µL volume which is added to each positive control plate. The stock solutions are stored frozen in aliquots at -90°C and thawed just before use.

S-9 Activation System

Prepare S-9 activation system immediately before use.

S-9 Activation System Co-factor Mix

To prepare 9.6 mL:

5 mL	0.2 M Sodium phosphate buffer, pH 7.4
0.2 mL	0.4 M $MgCl_2$ + 1.65 M KCl
0.5 mL	0.1 M Glucose-6-PO_4
0.4 mL	0.1 M NADP
3.5 mL	Milli-Q deionized water

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Complete S-9 Activation System

To prepare 10 mL:

The final S-9 activation system is prepared by adding 0.4 mL of freshly thawed Aroclor 1254-induced rat liver S-9 microsome preparation to 9.6 mL of the co-factor mix. The S-9 microsome preparation is either prepared in-house according to the method of Ames et al. (1975) or purchased commercially.

The S-9 activation system is thoroughly mixed, sterilized by passage through a membrane filter with a 0.45 µ pore size, and kept ice-cold until it is used. Any unused S-9 activation system is discarded at the end of the day.

PROCEDURE:

1. Bacterial Tester Strain Maintenance and Growth

1.1 Frozen Stocks for Preparing Master Plates

Bacteria from the frozen Master Copy stocks are grown overnight in Oxoid nutrient broth in a 37°C shaker water bath. These cultures are frozen in Oxoid nutrient broth containing 8% dimethylsulfoxide (DMSO) and are used only for making Master Plates.

1.2 Master Plates

The Master Plates are used as the source of the bacteria for the overnight cultures used in the mutagenicity test.

To prepare the agar for Master Plates, spread 0.1 mL of 0.1 M histidine and 0.5 mM biotin on the surface of minimal glucose agar plates using a sterile glass spreader. For the R-factor strains, TA98 and TA100, spread 0.1 mL of ampicillin (8 mg/mL) in addition to the histidine and biotin as pressure for retaining the plasmid. The plates should be prepared a day or two before use to allow these solutions to diffuse uniformly throughout the agar.

Grow up overnight, Oxoid nutrient broth cultures from the frozen permanents. Using a sterile wire loop, transfer some culture to the surface of the agar, making 5 or 6 parallel streaks across the plate. Incubate overnight at 37°C and store in the refrigerator. The plates can be used for 2-3 months before new Master Plates must be made.

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1.3 Overnight Cultures For Use In The Mutagenicity Test

Bacteria from the Master Plates are grown overnight in Oxoid nutrient broth in a 37°C shaker water bath. These cultures furnish the bacteria used in the mutagenicity test. The cultures are kept ice-cold while they are being used during the test.

2. Test Article Preparation

Test articles are weighed in a room illuminated with subdued light and the solutions are prepared in a room illuminated with yellow light. All test article solutions should be handled with a micropipetting system with polypropylene tips.

Test articles to be tested for mutagenicity are routinely dissolved in DMSO if they are insoluble in Milli-Q deionized water. For compounds which are also insoluble in DMSO, there is a need for other solvents which are non-toxic to the bacteria and to the microsomal enzymes in the S-9 mix. The following solvents are acceptable for use in the assay: Pure 95% ethanol at a maximum of 0.1 mL/plate, acetone, ethylene glycol dimethyl ether, formamide, dimethylformamide, 1-methyl-2-pyrrolidinone, glycerol formal, tetrahydrofurfuryl alcohol, tetrahydrofuran or acetonitrile. For routine tests, the test article solution is diluted with either Milli-Q deionized water or DMSO, as appropriate, so all the dose levels are contained in a 0.1 mL volume.

3. Cytotoxicity Dose-Range-Finding Test

In order to determine an appropriate range for the dose levels of the test article used in the test, the tester strain TA1538 is exposed to at least 8 dose levels of the test article in the presence and absence of the S-9 activation system following the assay protocol below (Section 4 below). At the end of the 48-hour incubation, the plates are examined microscopically for a cytotoxic effect of the test article as indicated by a sparse and/or unevenly distributed background lawn of bacterial growth. Based on the results of this test, five dose levels are chosen for use in the mutagenicity test.

4. Assay Protocol

4.1 Choice of Dose Range of the Test Article

Dose levels up to at least 5 mg/plate should be tested unless limitations are imposed by toxicity or solubility or other considerations, such as usage level, etc. A reasonable lower limit in routine testing is 0.2 µg/plate. The highest dose level tested should be

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5 mg/plate or, if the test article has been shown to be cytotoxic to the tester strains the dose range chosen should include at least one observably cytotoxic dose. Similarly, if preliminary solubility tests show that a test article precipitates at dose levels below 5 mg/plate, subsequent tests should include at least one dose at which the precipitate occurs. A total of five dose levels of the test article should be run unless specified otherwise.

4.2 Positive Controls

In order to determine the specific sensitivities of each tester strain to specific mutagens and to determine if the S-9 activation system is performing properly, the five positive control mutagens (see above) are run at the following dose levels contained in 10 µL of DMSO:

Test Strain	Positive Control Mutagen	µg/Plate
TA98	4-nitroquinoline-N-oxide	1
TA100	Benzo(a)pyrene	5
TA1535	N-methyl-N'-nitro-N-nitrosoguanidine	4
TA1537	9-Aminoacridine	100
TA1538	2-Aminofluorene	10

Benzo(a)pyrene and 2-Aminofluorene are run with S-9 activation and the other mutagens are run without metabolic activation.

The following are added (in order) to 2 mL molten top agar in a 20 x 125-mm sterile screw-cap glass tube: 0.1 mL (∿ 2 to 4 x 10^8 cells) of an overnight culture of the appropriate Salmonella tester strain, 10 µL of the mutagen solution and 0.5 mL of the S-9 activation system in those cultures to be run with metabolic activation. The contents of the tubes are quickly mixed on a Vortex mixer and immediately poured onto the surface of minimal glucose agar plates. Uniform distribution of the mixture on the surface of the plate agar is accomplished by gently tilting and rotating the uncovered plate. The top agar is allowed to harden for approximately 1/2 hour before the plates are placed in a dark 37°C incubator. All positive controls are run on triplicate plates.

4.3 Negative Solvent Controls

The negative solvent controls consist of a volume of the solvent, used to dissolve the test article, equal to the volume of the test article solution added to the molten top agar, as described below (see Section 4.5). The solvent controls are run both with and without S-9 activation system on triplicate plates. All five tester strains are used.

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4.4 Sterility Controls

The sterility of the S-9 activation system, the solvent and the test article solution is determined by mixing 0.1 mL of each with 2 mL of top agar and spreading the mixtures on the surface of separate *triplicate* minimal glucose agar plates.

The sterility of the top agar is determined by spreading 2 mL of the melted (45°C) top agar on the surface of *triplicate* minimal glucose agar plates.

The sterility plates are incubated for 2 days at 37°C and then examined for colony growth. The number of colonies, arising from contaminating organisms, is recorded on the data sheet.

4.5 Plate Incorporation Technique

The following are added (in order) to 2 mL molten top agar in a 20 x 125-mm sterile screw-cap glass tube: 0.1 mL (∿ 2 to 4 x 10^8 cells) of an overnight culture of the appropriate *Salmonella* tester strain, 0.1 mL of appropriately diluted test article solution, 0.5 mL of the S-9 activation sytem in those cultures to be run with metabolic activation. The contents of the tubes are quickly mixed on a Vortex mixer and immediately poured onto the surface of minimal glucose agar plates.

Uniform distribution of the top agar on the surface of the plate agar is accomplished by gently tilting and rotating the uncovered plate. The top agar is allowed to harden for approximately 1/2 hour before the plates are placed in a dark 37°C incubator.

All five *Salmonella* tester strains, TA100, TA98, TA1535, TA1537, and TA1538, with and without S-9 metabolic activation, are used.

All dose levels of the test article on each tester strain are performed on single, duplicate *or* triplicate plates, depending on the wishes of the sponsor.

4.6 Revertant Colony Counts

After 48 hours incubation at 37°C, the colonies (revertants to histidine prototrophy) on all test and control plates are counted, and the presence of the light background growth (background lawn), due to the trace amounts of histidine in the agar, is confirmed. The test plates are observed for the presence of a precipitate or cytotoxic effects. If they are present, they are noted on the data sheet.

The toxicity of some test articles may delay the appearance of revertants. When such a phenomenon is suspected, the test plates should be incubated for 72 hours. The client should be advised of this in advance of extending the incubation period. Counting of the colonies on the plates is accomplished with the aid of a Quebec Colony Counter and a Biotran II Automated Colony Counter. The Biotran II Automated Colony Counter is calibrated and operated according to Raltech Standard Operating Procedure OP-TOX 811.

The number of revertant colonies on each plate is entered in the appropriate space on the data sheet (see attachment). The average number of revertant colonies/plate is calculated and entered on the data sheet.

The number of revertant colonies on the positive mutagen and solvent control plates should be within the normal historical limits of variability.

4.7 Interpretation of Test Results

A test article is considered to have a negative mutagenic effect under the conditions of the test, if at least 5 mg (or the maximum nontoxic or non-precipitating dose level or the maximum dose level based on other considerations such as usage level, etc.) has been tested and the average total number of revertant colonies/plate* is less than two times the average number of spontaneous (background) revertant colonies/plate from the corresponding solvent control plates and/or there is no significant ($p > 0.05$) dose-response relationship. If necessary, a square root transformation of the data (Weinstein and Lewinson, 1978) is analyzed using analysis of variance techniques. Where significant ($p < 0.05$) differences are indicated among groups, treatment groups will be compared to the control group using Dunnett's test.

---

* Average total number of induced revertant colonies/plate plus the average number of spontaneous (background) revertant colonies/plate from corresponding solvent control plates.

REFERENCES:

Ames, B., J. McCann and E. Yamasaki, Mutation Research, 31, 347-364 (1975).

Weinstein, D. and T. Lewinson, Mutation Research, 51, 433-434 (1978).

APPROVED BY: Robert A. Finch DATE 10/10/80
Robert A. Finch, PhD
Head, Genetic Toxicology

REVIEWED BY: Stephen D. Morton DATE 10/10/80
Stephen D. Morton, Manager
Quality Assurance Unit

Ames Salmonella Mutagenicity Test Results

Plate Incorporation Test

Submitted by______________________________

Date Received_______________

Raltech Number_______________

Sample Description____

Positive Mutagen Controls	Salmonella Strain	Concentration µg/Plate	S-9 System Incorporated	Total Revertant Colonies Per Plate	Average
4-Nitroquinoline-1-oxide	TA98	1 *	-		
Benzo(a)pyrene	TA100	5 *	+		
N-methyl-N-nitro-N-nitrosoguanidine	TA1535	4 *	-		
9-Aminoacridine	TA1537	100 *	-		
2-Aminofluorene	TA1538	10 *	+		

		Spontaneous Revertant Colonies per Plate									
Untreated Controls	S-9	TA98	Ave.	TA100	Ave.	TA1535	Ave.	TA1537	Ave.	TA1538	Ave.
	- **										
	+ ***										

			Total Revertant Colonies per Plate									
Sample	µg/Plate	S-9	TA98	Ave.	TA100	Ave.	TA1535	Ave.	TA1537	Ave.	TA1538	Ave.
		-										
		-										
		-										
		-										
		-										
		+										
		+										
		+										
		+										
		+										

* Performed as Plate Incorporation Test
** No S-9 Activation System Incorporated in Test
*** S-9 Activation System Incorporated in Test

Sterility Controls (colonies/plate)

S-9 System________ Top Agar________

Solvent________ Sample________

Test Date____________

Date Read____________

Signed:____________

APPENDIX V

Laboratory: Research Triangle Institute
P.O. Box 12194
Research Triangle Park, NC 27709

Submitter: Dr. Thomas J. Hughes
Head, Ames Laboratory
(919)541-6148

Date of submission: December 15, 1980
updated July 29, 1981

Omissions: Appendices 2, 3, and 4

RESEARCH TRIANGLE INSTITUTE
POST OFFICE BOX 12194
RESEARCH TRIANGLE PARK, NORTH CAROLINA 27709

CHEMISTRY AND LIFE SCIENCES GROUP

December 15, 1980

Ms. Stephanie Toney
Genetic Bioassay Branch
MD68
U. S. EPA
Health Effects Research
  Laboratory
Research Triangle Park, NC 27711

Dear Ms. Toney:

Please find enclosed a copy of RTI's In Vitro Bacterial Mutagenesis Capability. My name is Thomas J. Hughes and I have replaced Dr. Kolber as head of RTI's Ames Laboratory.

Two of the major differences between our protocol and the Ames standard protocol are: (1) use of NADPH directly instead of an NADPH-generating system, and (2) a quantitative toxicity screen, which utilizes 1,000 colonies/plate under the identical conditions of the mutagenesis assay.

We use NADPH directly because the high toxicity of complex environmental samples can inhibit a NADPH-generating system. The quantitative toxicity assay is used to identify non-toxic dosages and therefore prevent the generation of false positive results due to high levels of histidine from lysed cells. We are presently updating this assay to more closely approximate conditions in the mutagenicity assay.

Please feel free to call me at 541-6148 if you have any questions. I am updating this capability statement and will send you a copy when available.

Sincerely,

Thomas J. Hughes

Thomas J. Hughes

TJH/tjh

(919) 541-6000 FROM RALEIGH, DURHAM AND CHAPEL HILL

RESEARCH TRIANGLE INSTITUTE

STANDARD OPERATING PROCEDURES MANUAL

AMES/SALMONELLA BIOASSAY

June 22, 1981

Thomas J. Hughes
Laboratory Supervisor

RESEARCH TRIANGLE PARK, NORTH CAROLINA 27709

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BACTERIAL BIOASSAY SYSTEM

The procedures for handling the strains and preparing media components are those of Ames et al. (Ames, B. N., J. McCann, and E. Yamasaki. Methods for Detecting Carcinogens and Mutagens with the Salmonella Mammalian Microsome Test, Mutation Research 31, 347-364, 1975). The S-9 microsomal preparation is obtained from rats injected with Aroclor 1254; protein is measured by the method of Lowry et al. (Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein Measurement with the Folin Phenol Reagent, J. Biol. Chem. 193, 265-275, 1951).

PROTOCOL FOR TESTING

The standard assay is divided into four parts as follows.

Toxicity Testing, Plate Incorporation Method (see Table 1)

Eight hundred cells per dish are plated using nonselective media (histidine-positive overlay). Tests are done with and without induced S-9. Test compound is added at 0.1 ml/plate in all tests. The viability ratio is calculated as the ratio of surviving colonies with sample, to colonies without sample. A value less than one indicates toxicity of the sample compound; a value of one or greater indicates no toxicity. All toxicity determinations are made in triplicate both with and without microsomal S-9. Bioassay quality control is accomplished using solvent controls, positive controls and strain controls, all with and without microsomal S-9.

Mutagenesis Testing, Plate Incorporation Method (see Table 2)

Cells are plated at 10^8 cells/plate using selective media (histidine-negative overlays). Tests are done with and without induced S-9. Test compound is added at 0.1 ml/plate in all tests. The mutagenic ratio is calculated as the ratio of surviving colonies with sample, to

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Table 1. Standard Protocol for Toxicity Determinations in Ames/Salmonella Bioassay

Added to 2.5 ml agar, histidine (+), before layering onto plates[a]

Lab Code ID and Tube No.[a]	Sample in Vehicle ml	S-9 Microsomes[b] per ml of S-9 Mix	S-9 Mix in H_2O ml	Activation Prep. Medium Phosphate Buffer ml	Vehicle ml	Bacteria ml (10,000 cells/ml)	
1-1	.1 (1000 µg)/plate	0.1	0.5	0	0	0.1	with induced activation fraction
1-2	.1 (500 µg)/plate	0.1	0.5	0	0	0.1	
1-3	.1 (250 µg)/plate	0.1	0.5	0	0	0.1	
1-4	.1 (100 µg)/plate	0.1	0.5	0	0	0.1	
1-5	.1 (10 µg)/plate	0.1	0.5	0	0	0.1	
1-6	.1 (1000 µg)/plate	0	0	0.5	0	0.1	without activation fraction
1-7	.1 (500 µg)/plate	0	0	0.5	0	0.1	
1-8	.1 (250 µg)/plate	0	0	0.5	0	0.1	
1-9	.1 (100 µg)/plate	0	0	0.5	0	0.1	
1-10	.1 (10 µg)/plate	0	0	0.5	0	0.1	
1-11	0	0	0	0.5	0.1	0.1	negative controls
1-12	0	0.1	0.5	0	0.1	0.1	
1-13	0	0	0	0.5	0	0.1	
1-14	0	0.1	0.5	0	0	0.1	
1-15	0	0	0	0	0	0.1	

[a] Each done in triplicate.

[b] In 0.15 M KCl.

Table 2. Standard Protocol for Mutagenesis Determinations in Ames/*Salmonella* Bioassay

Lab Code ID and Tube No.[a]	Added to 2.5 ml agar, histidine (-), before layering onto plates[a] Sample in Vehicle ml	S-9 Microsomes[b] per ml of S-9 Mix	S-9 Mix in H_2O ml	Activation Prep. Medium Phosphate Buffer ml	Vehicle ml	Bacteria ml (10^9 cells/ml)	
2-1	.1 (1000 μg)/plate	0.1	0.5	0	0	0.1	with induced activation fraction
2-2	.1 (500 μg)/plate	0.1	0.5	0	0	0.1	
2-3	.1 (250 μg)/plate	0.1	0.5	0	0	0.1	
2-4	.1 (100 μg)/plate	0.1	0.5	0	0	0.1	
2-5	.1 (10 μg)/plate	0.1	0.5	0	0	0.1	
2-6	.1 (1000 μg)/plate	0	0	0.5	0	0.1	without activation fraction
2-7	.1 (500 μg)/plate	0	0	0.5	0	0.1	
2-8	.1 (250 μg)/plate	0	0	0.5	0	0.1	
2-9	.1 (100 μg)/plate	0	0	0.5	0	0.1	
2-10	.1 (10 μg)/plate	0	0	0.5	0.1	0.1	
2-11	0	0	0	0	0.1	0.1	negative controls
2-12	0	0.1	0.5	0.5	0	0.1	
2-13	0	0	0	0	0	0.1	
2-14	0	0.1	0.5	0	0	0.1	
2-15	0	0	0	0	0	0.1	

---

[a] Each done in triplicate.

[b] In 0.15 M KCl.

colonies without sample. A value above 2.5 on a reproducible dose-response curve is considered to be mutagenic: any number below 2.5 is considered nonmutagenic. All mutagenic determinations are made in triplicate, both with and without microsomal S-9.

Positive Mutagen Control Testing, Plate Incorporation Method

Using histidine-negative overlays, ~10^8 cells are plated in each dish. Known mutagens are tested to assure that the strains are active and the S-9 preparation was activating promutagens to the desired levels. If known positive controls do not show proper mutagenic activity, the test components (cultures and/or S-9) are rejected. Control compounds currently in use are:

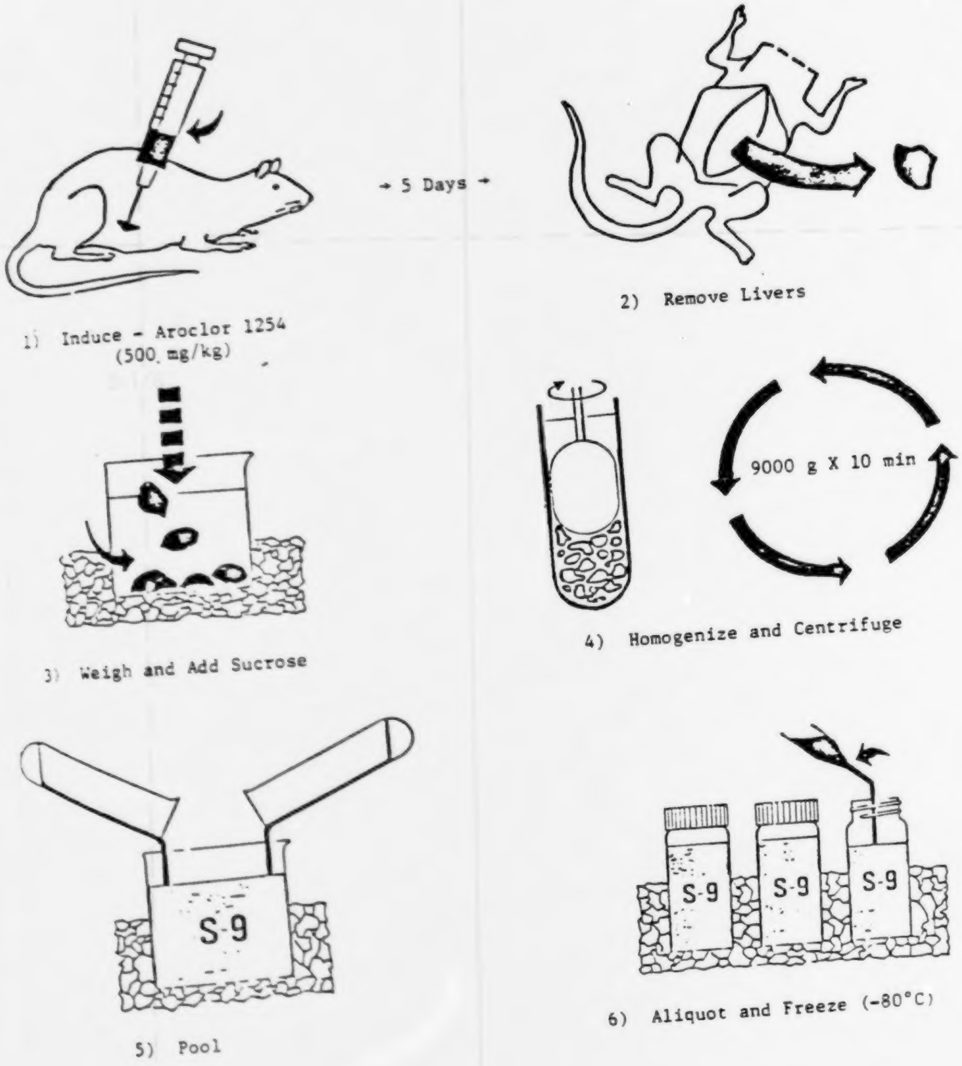
Strain	Without S-9	With S-9
TA 1535	Sodium azide 5, 1 μg/plate	2-Aminoanthracene 5, 2.5 μg/plate
TA 1537	9-Aminoacridine 100, 50 μg/plate	2-Aminoanthracene 10, 2.5 μg/plate
TA 1538 & TA 98	2-Nitrofluorene 10, 5 μg/plate	2-Aminoanthracene 5, 1 μg/plate
TA 100	Sodium Azide 5, 1 μg/plate	2-Aminoanthracene 5, 1 μg/plate

Tests are done in triplicate.

Sterility Testing, Plate Incorporation Method

Sterility tests are conducted with histidine-positive overlay plates, using the identical components employed in the tests. Components tested are sample(s), positive controls, solvent(s), water, 0.15 M KCl solution, microsomal preparation (S-9), S-9 mix, and agar plates. Sterility tests are done in duplicate.

PREPARATION of S-9



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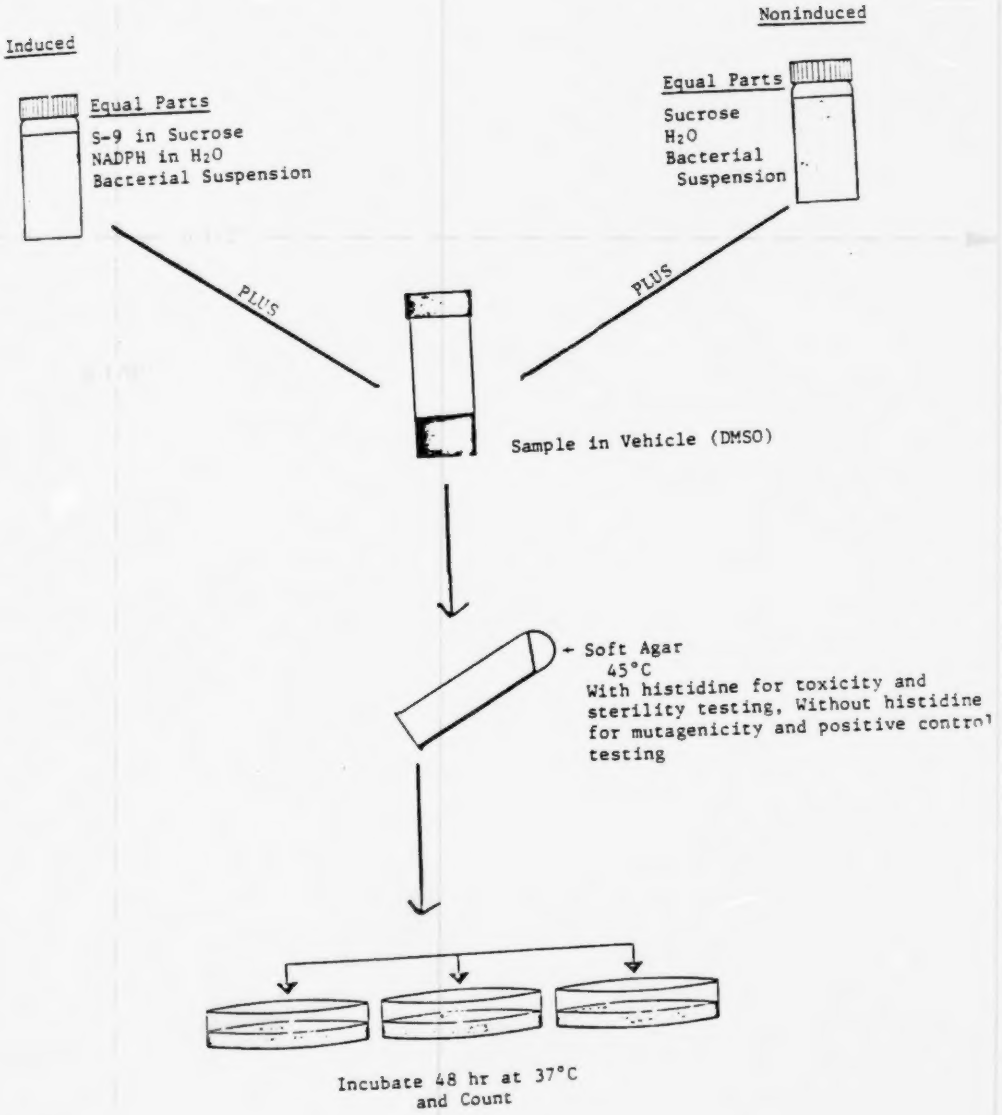
ASSAY PROTOCOL



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SAMPLE FORM

STRAIN: ____________ **PURPOSE:** ____________ **DATE** ____________

1. TOXICITY TEST

Bacteria: Strain ____________

420 O.D. ____________

hrs. in Culture ____________

RAT S-9: Type ____________

AM Age ____________

AM ____________

Vehicle: ____________

Comments: ____________

Total Amt. Microsomes Needed (All Tests) ____________

Compound: ____________

Concentration of Test Compound: ____________

2. MUTAGENIC TEST

Bacteria: Strain ____________

420 O.D. ____________

Comments: ____________

3. POSITIVE CONTROLS

Bacteria: Strain ____________

O.D. ____________

Compounds & Concentrations: ____________

Vehicle: ____________

Comments: ____________

4. STERILITY: ____________

5. RESULTS: ____________

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SAMPLE FORM

TOXICITY

Data Summary

Sample: ______________________________

Strain: ______________________________

Date: ______________________________

Sample Amount (ug/plate)	Lab Code	P1	P2	P3	Mean	Standard Deviation	Viability Ratio
	1-1						
	1-2						
	1-3						
	1-4						
	1-5						
	1-6						
	1-7						
	1-8						
	1-9						
	1-10						
	1-11						
	1-12						
	1-13						
	1-14						
	1-15						
	1-16						
	1-17						
	1-18						
	1-19						
	1-20						

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SAMPLE FORM

MUTAGENIC

Data Summary

Sample: ______________________

Strain: ______________________

Date: ______________________

Sample Amount (ug/plate)	Lab Code	F1	F2	F3	Mean	Standard Deviation	Mutagenic Ratio
	2-1						
	2-2						
	2-3						
	2-4						
	2-5						
	2-6						
	2-7						
	2-8						
	2-9						
	2-10						

SAMPLE FORM

POSITIVE CONTROL

Data Summary

Sample: ______________________________

Strain: ______________________________

Date: ______________________________

Sample Amount (ug/plate)	Lab Code	P1	P2	P3	Mean	Standard Deviation	Relative Mutagenic Ratio
	3-1						
	3-2						
	3-3						
	3-4						
	3-5						
	3-6						
	3-7						
	3-8						
	3-9						
	3-10						
	3-11						
	3-12						

SAMPLE FORM

STRAIN: TA 1535

DATE: ____________________

(3) POSITIVE CONTROLS - 3x

(1) 2AN (100) + AM
(2) 2AN (10) + AM
(3) 2AN (1) + AM
(4) 2AN (100) + NM
(5) SA (200) + NM
(6) SA (20) + NM
(7) SA (2) + NM
(8) ______________
(9) ______________
(10) ______________
(11) ______________
(12) ______________

---

(4) STERILITY - 2x

(1) AM
(2) NADPH
(3) Nutrient Broth
(4) Sucrose
(5) H_2O
(6) DMSO
(7) 2AN (100)
(8) SA (200)
(9) ______________
(10) ______________
(11) ______________
(12) ______________

SA = Sodium Azide
2AN = 2-Anthramine
AM = S-9 Microsomes added
2NF = 2-Nitrofluorene
NM = No S-9 Microsomes added
9AA = 9-Aminoacridine

SAMPLE FORM

STRAIN: TA 1537

DATE: ____________

(3) POSITIVE CONTROLS - 3x

(1) 2AN (100) + AM
(2) 2AN (10) + AM
(3) 2AN (1) + AM
(4) 2AN (100) + NM
(5) 9AA (250) + NM
(6) 9AA (100) + NM
(7) 9AA (50) + NM
(8) ____________
(9) ____________
(10) ____________
(11) ____________
(12) ____________

---

(4) STERILITY - 2x

(1) AM
(2) NADPH
(3) Nutrient Broth
(4) Sucrose
(5) H_2O
(6) DMSO
(7) 2AN (100)
(8) 9AA (250)
(9) ____________
(10) ____________
(11) ____________
(12) ____________

SA = Sodium Azide
2AN = 2-Anthramine
AM = S-9 Microsomes added
2NF = 2-Nitrofluorene
NM = No S-9 Microsomes added
9AA = 9-Aminoacridine

SAMPLE FORM

STRAIN: TA 1538

DATE: ____________

(3) POSITIVE CONTROLS -3x

(1) 2AN (100) + AM
(2) 2AN (10) + AM
(3) 2AN (1) + AM
(4) 2AN (100) + NM
(5) 2NF (100) + NM
(6) 2NF (10) + NM
(7) 2NF (1) + NM
(8) ____________
(9) ____________
(10) ____________
(11) ____________
(12) ____________

---

(4) STERILITY -2x

(1) AM
(2) NADPH
(3) Nutrient Broth
(4) Sucrose
(5) H_2O
(6) DMSO
(7) 2AN (100)
(8) 2NF (100)
(9) ____________
(10) ____________
(11) ____________
(12) ____________

SA = Sodium Azide
2AN = 2-Anthramine
AM = S-9 microsomes added
2NF = 2-Nitrofluorene
NM = No S-9 Microsomes added
9AA = 9-Aminoacridine

SAMPLE FORM

STRAIN: TA 98

DATE: ____________

(3) POSITIVE CONTROLS - 3x

(1) 2AN (100) + AM
(2) 2AN (10) + AM
(3) 2AN (1) + AM
(4) 2AN (100) + NM
(5) 2NF (100) + NM
(6) 2NF (10) + NM
(7) 2NF (1) + NM
(8) ____________
(9) ____________
(10) ____________
(11) ____________
(12) ____________

---

(4) STERILITY - 2x

(1) AM
(2) NADPH
(3) Nutrient Broth
(4) Sucrose
(5) H_2O
(6) DMSO
(7) 2AN (100)
(8) 2NF (100)
(9) ____________
(10) ____________
(11) ____________
(12) ____________

SA = Sodium Azide
2AN = 2-Anthramine
AM = S-9 Microsomes added
2NF = 2-Nitrofluorene
NM = No S-9 Microsomes added
9AA = 9-Aminoacridine

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SAMPLE FORM

STRAIN: TA 100 ________

DATE: ________________

(3) POSITIVE CONTROLS - 3x

(1) 2AN (100) + AM
(2) 2AN (10) + AM
(3) 2AN (1) + AM
(4) 2AN (100) + NM
(5) SA (200) + NM
(6) SA (20) + NM
(7) SA (2) + NM
(8) ____________
(9) ____________
(10) ____________
(11) ____________
(12) ____________

---

(4) STERILITY - 2x

(1) AM
(2) NADPH
(3) Nutrient Broth
(4) Sucrose
(5) H_2O
(6) DMSO
(7) 2AN (100)
(8) SA (200)
(9) ____________
(10) ____________
(11) ____________
(12) ____________

SA = Sodium Azide
2AN = 2-Anthramine
AM = S-9 Microsomes added
2NF = 2-Nitrofluorene
NM = No S-9 Microsomes added
9AA = 9-Aminoacridine

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TEST ORGANIZATION

Standardized data reporting formats are illustrated in Tables 3-5. A schematic of the test procedure is available in Figure 1. Included in assay organization are examples of Ames mutagenesis assay forms used for each experiment performed (Figures 2-8).

TEST COMPONENTS

BLUE OVERLAY 1 NM	1 AM
YELLOW OVERLAY 2 NM	2 AM
1 BACTERIA 1 Sucrose 1 H_2O	1 BACTERIA 1 AM (in sucrose) 1 NADPH (in H_2O)

METHODS

Toxicity Testing, Plate Incorporation Method

Cell Dilutions for Toxicity Testing

(1) Overlays are histidine-positive (Blue Caps)

(2) 700-1000 cells/plate are used.

O.D. Cells at 420 nm using the Gilford Spectrophotometer 2400. (OD should be between 1.2 and 1.5). MACHINE IS BLANKED TO ZERO using slit width (normal range between 0.07-0.08)

↓ 5 ml

1. 100 NB

↓ 2 ml

2. 100 NB

↓ 10 ml

3. 100 NB

↓ 20 ml

4. 100 NB $\xrightarrow[\text{0.1 ml/plate}]{\text{use}}$ 700-1000 cells/plate

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(3) Add the following in equal amounts to a scintillation vial:

With S-9 (Label 1 AM)	Without S-9 (Label 1 NM)
NADPH at 3.2 mg/ml in H_2O	H_2O
S-9 microsomal preparation in 0.25 M sucrose	Sucrose (0.25 M)
Bacterial Suspension	Bacterial Suspension

(4) Add 1.2 ml of the appropriate suspension from (3) to a dram vial and keep on ice.

(5) Add 0.4 ml of test material to the dram vial. Shake on virtus.

(6) Dispense 0.4 ml/2.5 ml overlay, shake and plate.

Mutagenesis Testing, Plate Incorporation Method

(1) Overlays are histidine-negative (Yellow Caps).

(2) Cells are not diluted.

(3) Add the following in equal amounts to a scintillation vial:

With S-9 (Label 2 AM)	Without S-9 (Label 2 NM)
NADPH at 3.2 mg/ml in H_2O	H_2O
S-9 microsomal preparation in 0.25 M Sucrose	0.25 M Sucrose
Bacterial Suspension	Bacterial Suspension

(4) Add 1.2 ml of the appropriate suspension from (3) to a dram vial and keep on ice.

(5) Add 0.4 ml of the test material to the dram vial. Shake on virtus.

(6) Dispense 0.4 ml/2.5 ml overlay, shake and plate.

Positive Mutagen Control Testing

A. ~10^9 cells are plated in each dish using His/neg. overlay (Yellow Caps).

B. Known mutagens are tested to assure

(1) strains are active

(2) microsomal prep is activating promutagens to desired levels.

C. If positive controls do not show proper activity or test components (culture and/or microsomes) are faulty, the test is rejected.

D. Positive controls to be used with proper strain and concentration according to the following scheme:

Strain	Without S-9*	With S-9*
TA 1535	Sodium Azide 200, 20, 2 μg/plate	2-Anthramine 100, 10, 1 μg/plate
TA 1537	9-Aminoacridine 250, 100, 50 μg/plate	2-Anthramine 100, 10, 1 μg/plate
TA 1538	2-Nitrofluorene 100, 10, 1 μg/plate	2-Anthramine 100, 10, 1 μg/plate
TA 98	2-Nitrofluorene 100, 10, 1 μg/plate	2-Anthramine 100, 10, 1 μg/plate
TA 100	Sodium Azide 200, 20, 2 μg/plate	2-Anthramine 100, 10, 1 μg/plate

*All samples are in 100 μl of DMSO

(1) Cells are not diluted

(2) Use the same scheme as described above for mutagenicity testing, substituting test material for positive control.

Sterility Testing

Sterility tests are conducted with histidine-positive overlay plates, using the amounts of components employed in the tests. Components tested are usually sample(s), positive controls, solvent(s), water, 0.25 M sucrose solution, microsomal preparation (S-9), and NADPH solution.

(1) Use histidine-positive overlay (Blue Cap).

(2) Dispense 0.1 ml of the appropriate component to be tested to two 2.5 ml overlays, shake, and plate.

Mutagenesis Testing, Spot Test Method

The spot test method is identical to the plate incorporation method (above), with the exception that, rather than being incorporated into the agar overlay, the test substance or positive control is placed on a filter disc (Whatman No. 1 filter paper) upon the surface of the overlay. For a test volume of 100 µl, a filter disk of 5 mm in diameter is used.

CHECKLIST

Day Before

1. Sterile - Biotips
   - Scintillation Vials
   - Dram Vials - Cold
   - Base Layer Plates
   - His +/- Overlays
   - 0.25 M Sucrose - Cold
   - Deionized H_2O - Cold
   - Nutrient Broth
   - Pipets
   - Spectrophotometer Cuvettes
2. Inoculate strains at 5:00 p.m. in cold nutrient broth (10 µl strain/50 ml NB). Always prepare duplicate flasks. Place on shaker.

Experiment Day

1. Spectrophotometer - warmed up.
2. Strain - place flasks on ice and O.D.
3. Water Baths - 45°C.
4. Overlays - +/- melted (autoclave 5 min liquids) and lowered to 45°C in water bath.
5. Protocol - written up.
6. Sample - weighed out and diluted in vehicle.
7. Positive Controls - remove from freezer and allow to thaw, or weigh out 20 mg in a sterile scintillation vial and dilute in vehicle.
8. Base Layer Plates - numbered and at 37°C.
9. Dram/Vials - numbered and at 4°C.
10. S-9 - take out of freezer, thaw and place on ice.
11. NADPH - remove from freezer, bring to room temperature, weigh out, calculate and dilute to 3.2 mg/ml in H_2O. Place on ice.
12. Incubators - evacuate.
13. Cells - dilute, keep on ice.

DATA HANDLING AND INTERPRETATION

The raw data is obtained as revertants/plate. Mean and standard deviations are calculated for replicate samples at each dose level and for positive and negative controls; mutagenic ratios are calculated for all sample concentration results (the viability is determined by cloning efficiency and must exceed 50%). The mutagenic ratio is defined as the average of triplicate plate counts at a given dosage divided by the average of triplicate plate counts for the appropriate solvent (blank) control. All results are tabulated on a standard printed format.

Several quality control checks and quality assurance procedures can be performed to insure the validity of data from Ames testing. Provisions for a complete, permanent, easily accessible record of the raw experimental data are made prior to, during, and following completion of the task. This includes a written record (in ink, in a bound, page-numbered notebook) of equipment serial numbers, reagents and supplies used, test data and a detailed record of any protocol modifications.

In addition to an assurance of careful and complete documentation, the raw Ames test data is subjected to the following internal evaluations for validity:

(1) B. Ames published normal ranges for spontaneous reversion and mutation levels with specific known mutagens (positive controls) for each of his tester strains. Results should fall within these ranges.

(2) Each dosage level is plated in triplicate. The standard deviation and mean ($\bar{X}$) of the plate count (numbers of revertants/plate) is calculated for each dosage level. Doses where the S.D. exceeds the mean by ± 25 are rejected. The same specifications apply for solvent, positive and negative controls and all test doses.

(3) Plate counts are considered valid only at dosage levels where quantitative toxicity testing indicates less than 50% of the bacteria were killed.

(4) Experiments where the components are contaminated or where the positive controls are not active, are rejected.

CALCULATIONS:

Toxicity Testing

A viability ratio is calculated as:

$$\frac{\text{no. colonies with sample}}{\text{no. of colonies without sample}} = \text{VIABILITY RATIO}$$

A viability ratio below 0.50 is an indication of a toxic effect of the sample compound; a viability ratio equal to or above one is an indication that the sample is nontoxic to the assay system (bacteria and S-9).

Mutagenic Testing

A mutagenic ratio is calculated as follows:

$$\frac{\text{no colonies with sample}}{\text{no. colonies without sample}} = \text{MUTAGENIC RATIO}$$

Any number above 2.5 on a reproducible dose-response curve is considered to be mutagenic: any number below 2.5 is considered nonmutagenic.

Mean number of revertants and mutagenic ratios standard deviations, are figured for each of the standard five dose levels (each dose is tested with triplicate plates).

PREPARATION OF TEST COMPONENTS

Base Layer Agar Plates

Difco Bacto Agar	24 gm
A-d-+ Glucose	30 gm
H_2O, distilled	1500 ml

Use two liter flask. Add stirring bar and autoclave for 30 min on liquids.

(1) Mix base layer agar medium and autoclave.

(2) Immediately after autoclaving, add 30 ml of sterile stock salt solution (S4) per 1500 ml of media.

(3) Maintain agar at 45°C for 30 min in water bath.

(4) Pour 25 ml aliquots into petri dishes (100 x 15 mm) using automatic plate pourer (Petrimat) set at 2.0.

(5) The following day, incubate up to two days at 37°C to check for contamination, and to dry. Re-bag, tape-close, and invert.

(6) Store under refrigeration in original box. Maximum storage is one month.

Stock Salt Solution (Vogel-Bonner Medium E) - S4*

	1X	3X
H_2O, deionized	670 ml	2010 ml
$MgSO_4 \cdot 7H_2O$	10 gm	30 gm
Citric acid$\cdot 1H_2O$	100 gm	300 gm
K_2HPO_4	500 gm	1500 gm
(Add 50 gm at a time with heat on 5)		
$NaNH_4HPO_4 \cdot 4H_2O$	175 gm	525

*Mix in 4 liter flask, ADD EACH chemical separately and in order. Do not add before previous chemical has gone into solution. Put 350 ml in each bottle: autoclave on liquids for 30 minutes. Can be stored tightly capped on the shelf indefinitely. Can also be dispensed in 30 ml aliquots.

Histidine-Negative Agar Overlay Media (Yellow Caps)

H_2O, deionized	1000 ml
NaCl	5 g
Bacto-Agar, purified	6 g

Add stir-bar and autoclave for 30 min on liquid cycle. Use two liter flask. Add 100 ml HIS/BIO solution after autoclaving.

Histidine-Positive Agar Overlay Media (Blue Caps)

H_2O, deionized	1000 ml
NaCl	5 gm
Bacto-Agar, purified	6 gm
L-histidine·HCl Monohydrate	48 mg

Add stir-bar and autoclave 30 min on liquids. Use two liter flask. Add 100 ml HIS/BIO solution after autoclaving.

His/Biotin Solutions

H_2O, deionized	500 ml
D-biotin	61.0 mg
L-histidine·HCl Monohydrate	48.0 mg

Calculate Total Liters of Overlay Media - Each Liter Requires 100 ml of HIS/BIO Solution - Autoclave for 30 minutes. Prepare and autoclave with +/- overlays and add immediately. DO NOT MAKE IN SMALLER QUANTITY TO AVOID WEIGHING MISTAKES. Discard excess.

GENERAL PROCEDURE

A. Autoclave all ingredients together with exception of His/biotin solution.

B. Immediately after autoclaving add sterile His/biotin solution (100 ml).

C. Cool to 45°C in water bath.

D. Dispense 2.5 ml aliquots into sterile glass test tubes using sterile Cornwall pipette.

E. Store in cold room indefinitely.

MEDIA

Oxiod Broth - Nutrient Broth

Broth	25 gm
H_2O, deionized	1000 ml

Put 50 ml/flask or 100 ml in brown bottles. Autoclave for 30 minutes on liquids. Store in cold room.

NB Agar Plates

Oxoid Broth	25 gm
Agar	16 gm
H_2O	1000 ml

Autoclave for 30 minutes on liquids. Place 25 ml/Petri dish, using automatic plate pourer (Petrimat) set at 2.0. Incubate overnight at 37°C to check for contamination, and to dry. Re-bag, tape closed, and invert. Store in cold room for up to two months.

1/4% Agar

Agar purified	250 mg
H_2O	100 ml

Autoclave for 30 minutes on liquids. Can be stored in cold room.

SOLUTIONS

NADPH (3.2 mg/ml)

H_2O	10 ml
Sigma NADPH	32.0 mg

Dissolve the NADPH by adding cold sterile H_2O in a large scintillation vial. Put on ice. Must be prepared daily as needed.

1.15% KCl (0.15 M KCl)

KCl	1.12 gm
H_2O, deionized	Bring up to 100 ml, bottle and autoclave - keep cold.

0.25 M Sucrose

Sucrose	85.6 gm/1000 ml
H_2O, deionized	(~950 ml H_2O), bring up to 1000 ml. Place in 100 + 30 ml bottles, autoclave, keep cold.

Can be stored tightly capped.

Saline Solution

H_2O, deionized	1000 ml
NaCl	8.5 gm

Place in appropriate bottles, autoclave 30 min on liquids. Can be stored at room temperature.

PREPARATION OF BACTERIA

Materials

A. Oxoid Broth

B. Nutrient Broth Agar

Procedure

A. Stock Cultures

(1) Grow up overnight cultures in oxoid broth (see B below)

(2) Add sterile DMSO (8.75% v/v) @ 4.4 ml/50 ml NB

(3) Place aliquots in 0.5 dram vials

(4) Freeze rapidly in dry ice/acetone

(5) Store at-80°C (Revco Freezer)

B. Working Cultures

(1) Using a stock culture, inoculate two 250 ml Erlenmeyer flasks with 10 µl of culture each containing 50 ml nutrient broth.

(3) Incubate at 37°C for 15 hr, shaking slowly (6.5). Start cold at 5:00 p.m. and remove following morning and place on ice.

(3) OD cells at 420 nm using Gilford Spectrophotometer (OD should be between 1.2-1.5)

(4) Concentrated cells at 420 nm are used for mutagenicity and positive control assays; cells are diluted using Nutrient Broth (NB) and established dilution sequence for toxicity assay.

PREPARATION OF S-9 FROM RAT LIVER MICROSOMES (1975)

The procedure of Ames et al., (1975) is used. Male Charles River rats, strain SD (200 g +/-20 g) serve as the source of liver material. The animals are housed one week before initiation of induction in suspended cages. Food is removed from the animal 12 hr before sacrifice. Water is given ad libitum. Induced rat liver (minimum of 20 rats) is obtained from rats injected intraperitoneally on day one with 500 mg/kg of Aroclor 1254 in Mazola® Corn Oil (0.5 ml of a 200 mg/ml for a 200 g rat). On day five, the animals are sacrificed with a guillotine. Liver is removed and placed in a sterile jar. Using sterile technique, livers are immersed in cold, sterile 0.25 M sucrose, washed two times, and blotted dry with sterile paper (Kimwipes). The livers are placed in a preweighed beaker, weighed, minced, and brought to 200 mg/ml with 0.25 M sucrose (3 ml/gm liver). The liver is homogenized (on ice) with four strokes of a cold Potter-Elvehjem apparatus with a Teflon pestle. The homogenate is centrifuged for ten minutes at 10,000 rpm using the Sorvall SS-34 rotor (9,000 g). The liver supernatant is poured in a sterile beaker and aliquoted. The stock solution is checked for sterility, quick-frozen in small aliquots (2-5 ml), in crytotubes and stored for a maximum of two months at -80°C (Revco Ultracold Freezer). For the assay, an aliquot of stock solution is slow-thawed and adjusted to the appropriate protein concentration in 0.25 M sucrose. NADPH is added at 0.32 mg/plate. Each batch is checked against known positive controls using the plate incorporation assay.

Injection Procedure For Rat S-9 (Ames Procedure)

1. Conc. of Aroclor 1254 in rat equals 500 mg/kg.
2. 150 gm rat gets 0.5 ml (i.p.) of a 150 mg/ml solution of Aroclor (1 ml Aroclor/9 ml corn oil).
3. 200 gm rat gets 0.5 ml (i.p.) of a 200 mg/ml solution of Aroclor (2 ml Aroclor/13 ml corn oil).
4. Aroclor 1254 = 1500 mg/ml solution.
5. Use syringe to draw up Aroclor.
6. Inject five days before sacrifice.
7. Math Formula

$$\frac{\text{Conc of Soln}}{\text{solvent (corn oil)}} = \text{resultant}$$

For 225 mg rat

i.e.: $\frac{1500 \text{ mg/ml}}{x \text{ ml}} = 225 \text{ mg/ml}$

1500 mg/ml = 225 mg/ml

1500/225 = x ml 6.66

dilution 1:6.66

mix 1 ml Aroclor to 5.66 ml corn oil.

8. ORDER AROCLOR FROM:

ANALABS
800 Republican Drive
North Haven, Conn.
Phone: (203) 288-8463

Wt. of Rat	Dilution	Resultant Concentration	Mixture Instructions	
			Aroclor	Corn Oil
150 grams	1:10.00	150 mg/ml	1 ml	9 ml
200 grams	1:7.50	200 mg/ml	1 ml	6.5 ml
225 grams	1:6.66	225 mg/ml	1 ml	5.66 ml
250 grams	1:6.00	250 mg/ml	1 ml	5.0 ml

9. Set up time for animal colony help for dav of harvest. 9:00 - 10:30 to 1:00 - 2:30.

S-9 Preparation Checklist

Day Before

1. Make sure rat food has been removed.

2. Sterilize -

   Scissors and forceps
   Pestles and tubes
   (4-6) Graduated cylinders (100 ml)
   (8-10) Beakers (500 ml)
   Disposable pipets (10 & 25 ml)
   Centrifuge tubes (6-8/batch)
   Kimwipes
   0.25 M Sucrose (500 ml/batch)
   Q-Tips
   Large mouth jars (10/batch)

3. Make nutrient agar plates - need 10.

Harvest Day

1. Need -

   Guillotine
   Paper towels
   Acetone
   Dry ice
   Plastic bags (large and small)
   Cryotubes
   Motor stand → HEX key
   Alcohol
   Medium and large gloves

2. Put note on centrifuge to reserve the appropriate times (10:00 a.m. to 12:00 p.m. and 2:00 p.m. to 4:00 p.m.).

3. Weigh 4-6 sterile beakers.

4. Make certain to keep the bodies separate from other refuse. Bodies are double bagged, put in cold room in animal colony and burned. Refuse is double bagged and put in cold room in Bldg. 3 and buried.

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WORKSHEET

Batch No. ____

____ Liver & Beaker Weight
- ____ Beaker Weight
____ Total Weight of Livers
x3
____ ml of sucrose to be added

Batch No. ____

____ Liver & Beaker Weight
- ____ Beaker Weight
____ Total Weight of Livers
x3
____ ml of sucrose to be added

Batch No. ____

____ Liver & Beaker Weight
- ____ Beaker Weight
____ Total Weight of Livers
x3
____ ml of sucrose to be added

Batch No. ____

____ Liver & Beaker Weight
- ____ Beaker Weight
____ Total Weight of Livers
x3
____ ml of sucrose to be added

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EPA-287 (Cin.)
(4-76)

SALMONELLA STRAIN VALIDATION (from Ames' Methods Paper, 1975).

The cells are taken from oxoid broth inoculated and grown overnight.

1. Histidine Requirement

   Base Layer Plates, total needed = 30
   5 strains - .1 ml/plate

   Overlays - H^+ (1A) and H^- (1B)

   A. Cells should grow with addition of histidine, heavy cloudy background.
   B. Spontaneous Background - must be within acceptable limits.

2. Crystal Violet/rfa Character

   Base Layer Plates, total needed = 15
   5 strains - .1 ml/plate

   Overlays - H^+
   10 µl of crystal violet on filter paper disc
   Place disc + cv on plate after pouring overly + cells

   A clear zone of inhibition should be present around the disc indicating that rfa cell wall mutation is present.

3. Ampicillin Resistant R Factor (Qualitative)

   Base Layer Plates, total needed = 15
   5 strains - .1 ml/plate

   H^+ overlays
   Pour overlays and cells; using q-tip, streak fresh ampicillin down middle of plate
   (Ampicillin - 100 µl of 8 mg/ml in .02 N NaOH)

   Strains 98 and 100 - No zone of inhibition around ampicillin streak
   Strains 37, 35, 38 - Zone of inhibition around ampicillin streak

4. UV Sensitivity/uvrB Deletion

   Base Layer Plates, total needed = 15
   5 strains - .1 ml/plate

   Overlays - H^+
   Pour plates, irradiate plats for
   - Strains 98 and 100 - 8 sec
   - Strains 35, 37, 38 - 6 sec
   - Using screen with T - cut out

   Cells should be present only on non-irradiated area.

5. Ampicillin Resistant R Factor (Quantitative Assay)

Base Layer Plates, total needed = 15
5 strains - 0.1 ml/plate after dilution in NB
5/100 → 1/100 → 10/100 → 20/100)

Overlays - H^+
Place 0.1 ml of diluted cells and 100 ~~µl~~ ampicillin in H^+ overlay
Strains 98 and 100 - full growth
Strains 35, 37, and 38 - no growth

6. Control for Ampicillin

Same as as #5, but no ampicillin is used.

7. Sterility

Plates - 3 each

Overlays - H^+ - 3 each

Overlays - H^- - 3 each

Acceptable Spontaneous Background Rates For Salmonella Strains (deserres and Shelby, 1979).

	Range	Ideal
TA 1535	5-50	20
TA 1537	3-25	7
TA 1538	5-40	25
TA 98	15-75	40
TA 100	60-220	160

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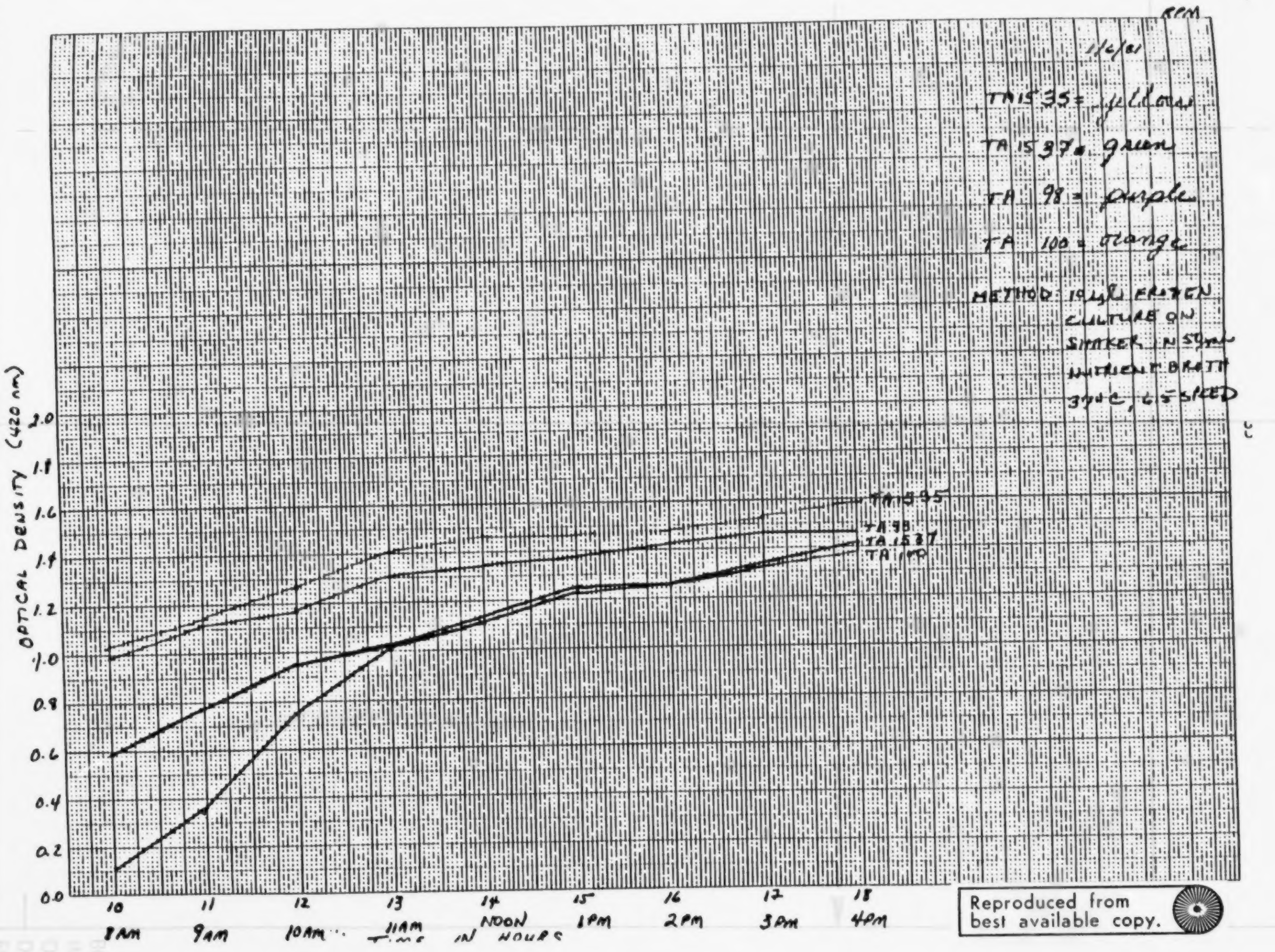

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METHOD: 10 µl FROZEN CULTURE ON SHAKER IN 50 ml NUTRIENT BROTH 37°C, 6.5 SPEED

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(4-76)

Tester Strains Quality Control Chart

STRAIN							
TA 98							
TA 100							
TA 1535							
TA 1537							
TA 1538							

QUALITY CONTROL FOR PROPER METABOLIC ACTIVATION FOR S-9

After each S-9 batch is prepared, metabolic activation is determined as follows: using four S-9 protein concentrations (0.8, 1.5, 3.0, 6.0 mg protein/plate) and two mutagens [Benzo(a)pyrene, 7,12-dimethylbenzanthracene] at five concentrations (100, 50, 25, 10, 1 µg/plate) and appropriate controls for each S-9 lot, the microsomal mediated mutagenicity for each S-9 preparation is determined. Each dose-level is performed in triplicate.

This experiment provides historical comparison data for metabolic activation, since S-9 preparations can vary in activity. A mutagenic ratio of at least 3.0 and a linear dose-response curve must be produced for each compound or the S-9 preparation is discarded. In general, our quality assurance and control requirements are in agreement with those suggested by deSerres and Shelby (deSerres, F., and M. Shelby, The Salmonella Mutagenicity Assay: Recommendations. Science, 203:563-565, 1979).

FORMULATION OF NADPH GENERATING SYSTEM

	Components	M.W.	Concentration/ml S9 Mix
1.	TPN	765.4	4 µmoles
2.	G6P	282	5 µmoles
3.	$MgCl_2$	203.3	8 µmoles
4.	KCl	74.5	33 µmoles
5.	Sodium phosphate buffer pH 7.4		100 µmoles
6.	Organ homogenate (S9 fraction)		100 µliters

Make-up following stock solutions:

0.2 M TPN	Millipore filter
0.2 M G6P	Millipore filter
0.4 M $MgCl_2$	Sterilize by autoclaving
1.65 M KCl	Sterilize by autoclaving
0.2 M Na Phosphate buffer pH 7.4	Sterilize by autoclaving

A. 2.0 ml and 0.2 ml aliquots of 0.2 M TPN is dispensed into sterile vials and kept frozen at -80°C until used.

B. 2.5 ml and 0.5 ml aliquots of 0.2 M G6P is dispensed into sterile vials and kept frozen at -80°C until used.

C. Mix the following and store at 4°C.

0.4 M MgCl	20 ml
1.65 M KCl	20 ml
0.2 M PB pH 7.4	500 ml
Sterile Distilled H_2O	315 ml

D. Make the core reaction mixture by adding:

0.2 M TPN	2.0 ml
0.2 M G6P	2.5 ml
Solution C.	85.5
	90.0 ml Filter Sterilize

Solution D should be prepared each day and all components should be kept ice cold before and after mixing.

AMES ACTIVATION SYSTEM

	Component	M	MW	Supplier	Stock Preparation	Volume of Stock added/ml of Final Mix	Per 100 ml	Final Concentration of Component/ml in Mix
1.	TPN[a]	0.2	765.4	ICN	76.6 g/500 mg H_2O	20 l	2.0	4 moles
2.	Glucose-6-phosphate dibasic	0.2	282	Sigma	28.2 g/500 ml H_2O	25 l	2.5	5 moles
3.	Sodium[b] phosphate	0.2	142 Di 138 Mono	Sigma	2 liter dibasic[c] 300 ml mono[d]	500 l	50.0	100 moles
4.	$MgCL_2$	0.4	203.3	Sigma	40.7 g/500 ml H_2O	20 l	2.0	8 moles
5.	KCL	1.65	74.5	Sigma	61.5 g/500 ml H_2O	20 l	2.0	33 moles
6.	Homogenate		-	-	Standard KCl 9,000 x g supernatant	100 l	10.0	Approximately 25 mg of fresh tissue equivalant
7.	H_2O					315 l	31.5	

[a] AM mix is 1-7 with the addition of bacteria.

[b] Sodium phosphate is used as AM mix plus bacteria, pH 7.4.

[c] 2 liter dibasic = 56.8 mg/200 ml.

[d] 300 ml mono = 8.28 gm/300 ml.

Components 1 and 2 are prepared in sterile distilled water and maintained at -20°C.

Components 3, 4, and 5 are prepared in distilled water, sterilized, and maintained at 4°C.

Components 6 is prepared and stored at -80°C until used.

Components 1-5 combined = core reaction mixture. MW = Molecular weight.

Components 1-6 combined = complete S-9 mixture.

Preparation of S-9 Mix: to 90.0 ml of Solution D add 10 ml of Organ Homogenate (S-9) to make up 100 ml of S-9 reaction mixture (Solution E). If needed filter sterilize. Both, Solution D and solution E should be kept cold before and after mixing. S-9 reaction mixture should be used the same day it is prepared. DISCARD UNUSED PORTION.

TOTAL PROTEIN ASSAY

Procedure of Lowry et al., J. Biol. Chem. 193, 265 (1951) modified slightly as regards the reagents. All reagents should be freshly prepared.

REAGENTS:

1. 2% Na_2CO_3 in 0.10 N NaOH

   0.1 N NaOH - 4.0 gm/1000 ml - then
   2.0 g Na_2CO_3/1000 ml of 0.1 N NaOH

2. 1% $CuSO_4 \cdot 5\ H_2O$ in H_2O - 1.0 g/100 ml H_2O

3. 2% NaK Tartrate (or Na or K) in H_2O - 2.0 gm/100 ml

4. Diluted Folin Reagent -- dilute Folin-Ciocalteau reagent (Fischer-Chem. Co.) 1:1 with H_2O. Store in amber bottle at 4°C.

5. Protein Standard - prepare a dilution series of Bovine Serum Albumin in H_2O from 2.0 mg/ml to 0.02 mg/ml. Store in tubes at 4°C. Will last for six weeks. Every six weeks a new standard series should be made.

6. Alkaline Cu Reagent - prepare just prior to each run. To each 100 ml of the Na_2CO_3 add 1.0 ml of the NaK Tartrate and mix. Then slowly add 1.0 ml $CuSO_4$ while continuing to stir to avoid precipitation of $Cu(OH)_2$.

PROCEDURE:

Combine: 1. .1 ml sample
2. .5 ml H_2O
3. 2.0 ml alkaline Cu reagent

Mix well and let stand at room temperature for at least 10 minutes or for as long as several hours.

Then add .2 ml dilute Folin reagent with immediate vigorous stirring.

Let stand 30 minutes at room temperature.

Read at 750 mu against a reagent blank.

(Reagent blank consists of exactly the same ingredients as a sample only, substitution of water for the sample).

NOTES:

1. The entire standard curve for the protein concentration is necessary since the color formation does not exactly follow Beer's law.

2. Vigorous mixing immediately on the addition of the Folin reagent is critical.

3. Aliquots should be run in triplicate including the standards.

4. The color obtained represents soluble cellular protein. Assays obtained by other procedures (biuret etc.) give somewhat higher values, presumably due to cell wall protein which does not appear to be completely determined by the Folin procedure. However, the other procedures are not as reproducible and are more tedious.

PRIORITY SCHEME FOR MUTAGENIC TESTING OF AIR PARTICULATE SAMPLES

In order to get the maximum amount of information with limited quantities of test fractions, the following order of priorities is suggested, the most important being listed first.

(1) 4 Strains, with the well test and with induced S-9 at two dose levels (100 and 500 μg/plate). PNA fractions to be done using pour plate, 500 μg, induced microsomes and TA 98.

(2) As in 1, but with 5 strains.

(3) Pour plate tests using induced S-9, and then no S-9, at 500 μg/plate.

(4) As in 3, but with 2 or more test concentrations (i.e., 1000, 500, 250, 100, 10 μg/plate. Preferred test concentrations are underlined.

(6) Strains in order of decreasing importance are: TA 98, TA 1535, TA 1537, TA 1358, TA 100 - well test; / TA 98, TA 100, TA 1535, TA 1537, TA 1538, - pour plate test.

In all cases test will be conducted in duplicated. Depending on amount of sample available as many as possible of the tests will be conducted. Only one concentration of the sample fraction will be made for all these tests. To obtain lower test concentrations, less sample volume per plate will be used.

Key

(1) Conduct well test (1 then 2) (except PNA fraction).

(1a) After well test go to 2.

(2a) Available sample is ≤ 10 mg.

Limited pour plate testing is possible, as complete well testing uses approximately 6-10 mg of sample.

(2b) Available sample is >10 <20 mg.

Do pour plate test with 2 strains (TA 98 and TA 100) at 2 or more doses (see 4 in priority scheme).

(2c) Available sample is ≥ 20 mg.

Do pour plate tests with 5 strains at five dose levels (see 4 in priority scheme) with induced S-9 and at varying concentrations of S-9 (double dose response curve).

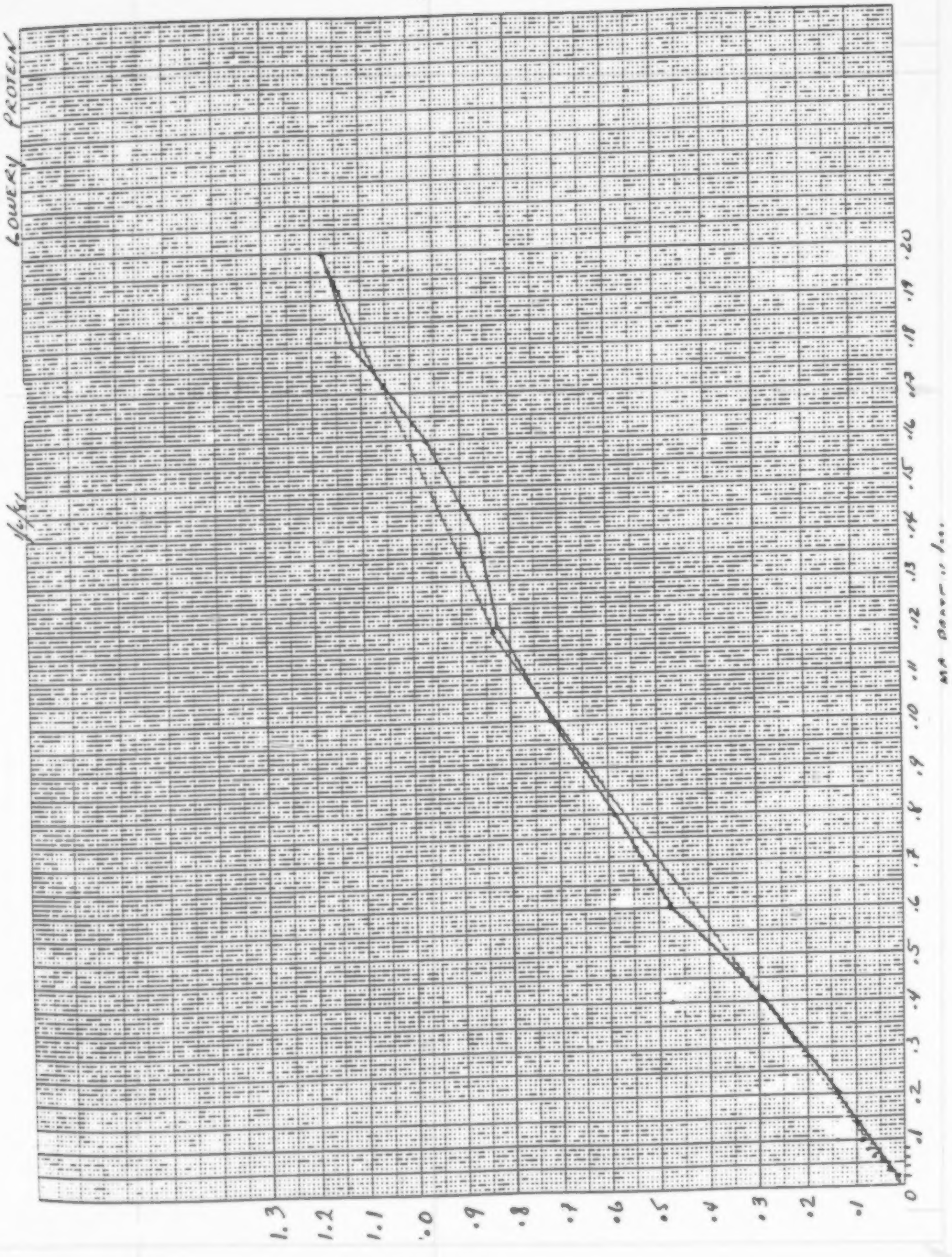
(2d) As in 2c but with no S-9.

(3) Repeat well test on positives, using the highest dose level (500 μg) and 2 strains. For positives, go to 4.

(4) Repeat pour plate tests on positives.

It is estimated that complete mutagenic testing will require 6.0-7.0 g of the total crude sample in addition to that consumed in the fractionation and chemical characterization. It will be necessary to have a minimum of 50 mg of each fraction to be tested. In addition, it would be desirable to have a portion of the sample stored for future reference.

When possible, toxicity testing will be done using strains TA 98 and TA 1535 with well plates at 500 μg/plate and with 1000 colonies/plate.


LOWERY PROTEIN
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MG PROT / ml

APPENDIX 1

NCI Procedures

CHECKLIST

Day Before

1. Sterile - Biotips
   - Scintillation Vials
   - Dram Vials - Cold
   - Base Layer Plates
   - His +/- Overlays
   - Sodium phosphate
   - Solution C
   - Nutrient Broth
   - Pipets
   - Spectrophotometer Cuvettes
2. Inoculate strains at 5:00 p.m. in cold nutrient broth (10 µl strain/50 ml NB). Always prepare duplicate flasks. Place on shaker.

Experiment Day

1. Spectrophotometer - warmed up.
2. Strain - place flasks on ice and O.D.
3. Protocol - written up.
4. Sample - weighed out and diluted in vehicle.
5. Positive Controls - weigh out new, and dilute in vehicle.
6. Water Baths - 45°C.
7. Overlays - +/- melted (autoclave 5 min liquids) and brought to 45°C in water bath.
8. Base Layer Plates - numbered and at 37°C.
9. Dram/Vials - numbered and cold.
10. S-9 - take out of freezer, thaw and place on ice.
11. Complete AM Mix - place on ice.
12. Incubators - evacuate.
13. Cells - dilute, keep on ice.

FORMULATION OF NADPH GENERATING SYSTEM

	Components	M.W.	Concentration/ml S9 Mix
1.	TPN	765.4	4 µmoles
2.	G6P	282	5 µmoles
3.	$MgCl_2$	203.3	8 µmoles
4.	KCl	74.5	33 µmoles
5.	Sodium phosphate buffer pH 7.4		100 µmoles
6.	Organ homogenate (S9 fraction)		100 µliters

Make-up following stock solutions:

0.2 M TPN	Millipore filter
0.2 M G6P	Millipore filter
0.4 M $MgCl_2$	Sterilize by autoclaving
1.65 M KCl	Sterilize by autoclaving
0.2 M Na Phosphate buffer pH 7.4	Sterilize by autoclaving

A. 2.0 ml and 0.2 ml aliquots of 0.2 M TPN is dispensed into sterile vials and kept frozen at -80°C until used.

B. 2.5 ml and 0.5 ml aliquots of 0.2 M G6P is dispensed into sterile vials and kept frozen at -80°C until used.

C. Mix the following and store at 4°C.

0.4 M MgCl	20 ml
1.65 M KCl	20 ml
0.2 M PB pH 7.4	500 ml
Sterile Distilled H_2O	315 ml

D. Make the core reaction mixture by adding:

0.2 M TPN	2.0 ml	
0.2 M G6P	2.5 ml	
Solution C.	85.5	
	90.0 ml	Filter Sterilize

Solution D should be prepared each day and all components should be kept ice cold before and after mixing.

NCI
Ames Activation System

	Component	M	MW	Supplier	Stock Preparation	Volume of Stock added/ml of Final Mix	Per 100 ml	Final Concentration of Component/ml in Mix
1.	TPN[a]	0.2	765.4	ICN	76.6 g/500 mg H_2O	20 1	2.0	4 moles
2.	Glucose-6-phosphate dibasic	0.2	282	Sigma	28.2 g/500 ml H_2O	25 1	2.5	5 moles
3.	Sodium[b] phosphate	0.2	142 Di 138 Mono	Sigma	2 liter dibasic[c] 300 ml mono[d]	500 1	50.0	100 moles
4.	$MgCl_2$	0.4	203.3	Sigma	40.7 g/500 ml H_2O	20 1	2.0	8 moles
5.	KCL	1.65	74.5	Sigma	61.5 g/500 ml H_2O	20 1	2.0	33 moles
6.	Homogenate		-	-	Standard KCl 9,000 x g supernatant	100 1	10.0	Approximately 25 mg of fresh tissue equivalant
7.	H_2O					315 1	31.5	

[a] AM mix is 1-7 with the addition of bacteria.

[b] Sodium phosphate is used as HM mix plus bacteria, pH 7.4.

[c] 2 liter dibasic = 56.8 mg/200 ml.

[d] 300 ml mono = 8.28 gm/300 ml.

Components 1 and 2 are prepared in sterile distilled water and maintained at -20°C.

Components 3, 4, and 5 are prepared in distilled water, sterilized, and maintained at 4°C.

Components 6 is prepared and stored at -80°C until used.

Components 1-5 combined = core reaction mixture. MW = Molecular weight.

Components 1-6 combined = complete S-9 mixture.

Preparation of S-9 Mix: to 90.0 ml of Solution D add 10 ml of Organ Homogenate (S-9) to make up 100 ml of S-9 reaction mixture (Solution E). Both, Solution D and solution E should be kept cold before and after mixing. S-9 reaction mixture should be used the same day it is prepared. DISCARD UNUSED PORTION. Solution E is complete AM mix.

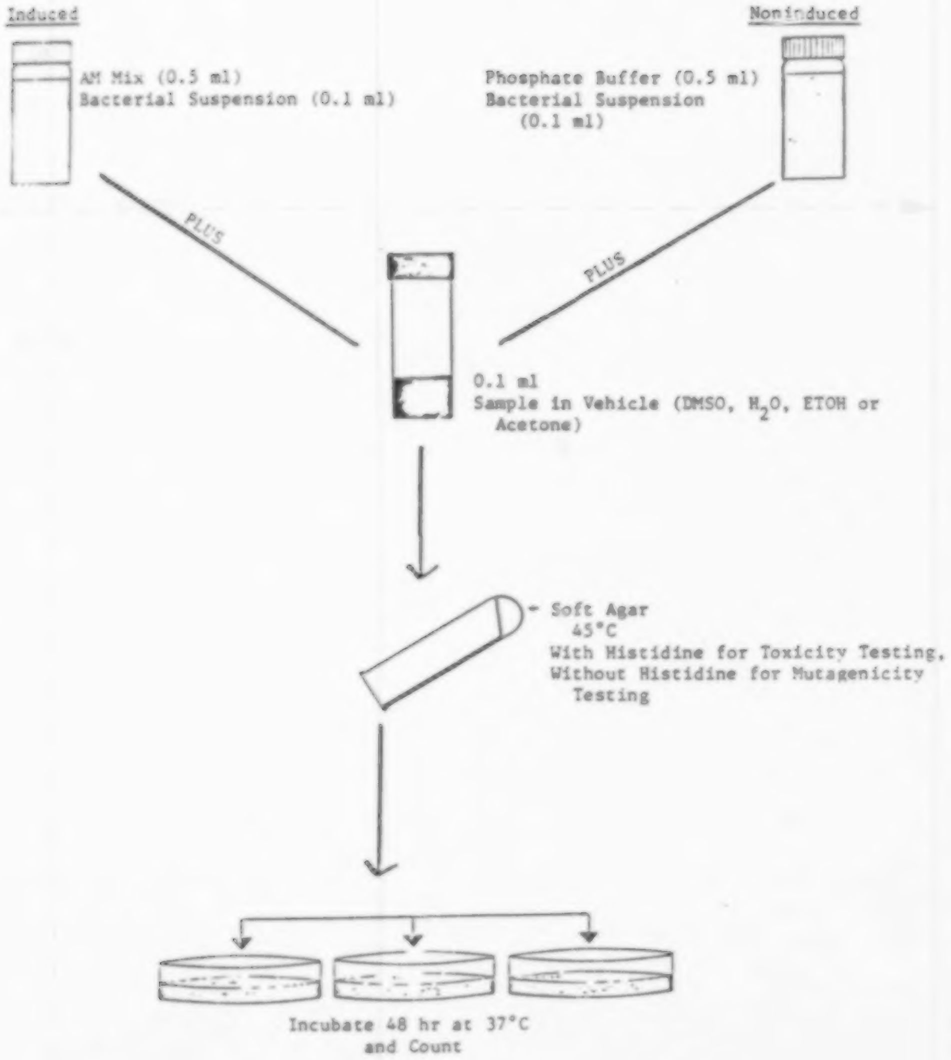
WORKSHEET

		Dispense
1.	4 gm TPN/26.1 ml H_2O	20 - 0.2 ml 11 - 2 ml
2.	20 gm G-6-P/354.6 ml H_2O	120 - 2.5 ml 100 - 0.5 ml
3.	85.2 gm sodium phosphate/3 ℓ H_2O adjust to 7.4 1500 ml needed	3 - 500 ml
4.	40.7 g magnesium chloride/500 ml H_2O 60 ml needed	4 - 100 ml
5.	61.5 g potassium chloride/500 mg H_2O 60 ml needed	4 - 100 ml

To make (Solution C) use --

500 ml #3	1500 ml	
20 ml #4 x 3	60 ml	
20 ml #5 x 3	60 ml	20 - 100 ml
315 ml H_2O x 3	945 ml	56 - 10 ml
	2565 TOTAL	

NCI AMES PROTOCOL


Induced
AM Mix (0.5 ml)
Bacterial Suspension (0.1 ml)
Noninduced
Phosphate Buffer (0.5 ml)
Bacterial Suspension (0.1 ml)
PLUS
PLUS
0.1 ml
Sample in Vehicle (DMSO, H2O, ETOH or Acetone)
Soft Agar
45°C
With Histidine for Toxicity Testing,
Without Histidine for Mutagenicity Testing
Incubate 48 hr at 37°C
and Count

Toxicity and Mutagenicity Preparation

1. Add 2 ml of the appropriate suspension to a dram vial and keep on ice.
2. Add 0.3 ml of the test material to the dram vial. Shake on virtus.
3. Dispense 0.7 ml/2.5 ml overlay, shake and plate.

TEST ORGANIZATION

Standardized data reporting formats are illustrated in Tables 3-5. A schematic of the test procedure is available in Figure 1. Included in assay organization are examples of Ames mutagenesis assay forms used for each experiment performed (Figures 2-8).

TEST COMPONENTS

BLUE OVERLAY 1 NM	1 AM
YELLOW OVERLAY 2 NM	2 AM
1 BACTERIA 5 Sodium Phosphate	1 BACTERIA 5 AM mix

METHODS

Toxicity Testing, Plate Incorporation Method

Cell Dilutions for Toxicity Testing

(1) Overlays are histidine-positive (Blue Caps)

(2) 700-1000 cells/plate are used.

O.D. Cells at 420 nm using the Gilford Spectrophotometer 2400. (OD should be between 1.2 and 1.5). MACHINE IS BLANKED TO ZERO using slit width (normal range between 0.07-0.08)

↓ 5 ml

1. 100 NB

↓ 2 ml

2. 100 NB

↓ 10 ml

3. 100 NB

↓ 20 ml

4. 100 NB $\xrightarrow[\text{0.1 ml/plate}]{\text{use}}$ 700-1000 cells/plate

Positive Controls For NCI

	AM	NM	Vehicle
TA 1535	2AN - 5, 2.5	SA - 5, 1	2AN-DMSO, SA-H_2O
TA 1537	2AN - 10, 2.5	9AA - 100, 50	2AN-DMSO, 9AA-ETOH
TA 1538	2AN - 5, 1	2NF - 10, 5	DMSO
TA 98	2AN - 5, 1	2NF - 10, 5	DMSO
TA 100	2AN - 5, 1	SA - 5, 1	2AN-DMSO, SA-H_2O

TYPING GUIDE SHEET

SAMPLE FORM

STRAIN: TA 1538

DATE: ____________________

(3) POSITIVE CONTROLS - 3x

(1) 2AN (5) + AM
(2) 2AN (1) + AM
(3) 2AN (5) + NM
(4) 2NF (10) + NM
(5) 2NF (5) + NM
(6) DMSO + AM
(7) DMSO + NM
(8) AM
(9) NM
(10) TA 1538
(11) ______________
(12) ______________
(13) ______________
(14) ______________
(15) ______________
(16) ______________
(17) ______________
(18) ______________

---

(4) STERILITY - 2x

(1) AM Mix
(2) NM Mix
(3) Nutrient Broth
(4) H_2O
(5) DMSO
(6) 2AN (5)
(7) 2NF (10)
(9) ______________
(10) ______________
(11) ______________
(12) ______________

SA = Sodium Azide
2AN = 2-Anthramine
AM = S-9 Microsomes added
2NF = 2-Nitrofluorene
NM = No S-9 Microsomes added
9AA = 9-Aminoacridine

SAMPLE FORM

STRAIN: TA 1537

DATE: ____________________

(3) POSITIVE CONTROLS - 3x

(1) 2AN (10) + AM
(2) 2AN (2.5) + AM
(3) 2AN (10) + NM
(4) 9AA (100) + NM
(5) 9AA (50) + NM
(6) DMSO + AM
(7) DMSO + NM
(8) AM
(9) NM
(10) TA 1537
(11) ______________
(12) ______________
(13) ______________
(14) ______________
(15) ______________
(16) ______________
(17) ______________
(18) ______________

---

(4) STERILITY - 2x

(1) AM Mix
(2) NM Mix
(3) DMSO
(4) ETOH
(5) 2AN (10)
(6) 9AA (100)
(7) ______________
(8) ______________
(9) ______________
(10) ______________

SA = Sodium Azide
2AN = 2-Anthramine
AM = S-9 Microsomes added
2NF = 2-Nitrofluorene
NM = No S-9 Microsomes added
9AA = 9-Aminoacridine

SAMPLE FORM

STRAIN: TA 1535

DATE: ____________________

(3) POSITIVE CONTROLS - 3x

(1) 2AN (5) + AM
(2) 2AN (2.5) + AM
(3) 2AN (5) + NM
(4) SA (5) + NM
(5) SA (1) + NM
(6) DMSO + AM
(7) DMSO + NM
(8) AM
(9) NM
(10) TA 1535
(11) ______________
(12) ______________
(13) ______________
(14) ______________
(15) ______________
(16) ______________
(17) ______________
(18) ______________
(19) ______________
(20) ______________

---

(4) STERILITY - 2x

(1) AM Mix
(2) NM Mix
(3) DMSO
(4) 2AN (5)
(5) SA (5)
(6) ______________
(7) ______________
(8) ______________
(9) ______________

SA = Sodium Azide
2AN = 2-Anthramine
AM = S-9 Microsomes added
2NF = 2-Nitrofluorene
NM = No S-9 Microsomes added
9AA = 9-Aminoacridine

SAMPLE FORM

STRAIN: TA 100

DATE: ____________________

(3) POSITIVE CONTROLS - 3x

(1) 2AN (5) + AM
(2) 2AN (1) + AM
(3) 2AN (5) + NM
(4) SA (5) + NM
(5) SA (1) + NM
(6) DMSO + AM
(7) DMSO + NM
(8) AM
(9) NM
(10) TA 100
(11) ______________
(12) ______________
(13) ______________
(14) ______________
(15) ______________
(16) ______________
(17) ______________
(18) ______________
(19) ______________
(20) ______________

---

(4) STERILITY - 2x

(1) AM Mix
(2) NM Mix
(3) DMSO
(4) 2AN (5)
(5) SA (5)
(6) ______________
(7) ______________
(8) ______________
(9) ______________

SA = Sodium Azide
2AN = 2-Anthramine
AM = S-9 Microsomes added
2NF = 2-Nitrofluorene
NM = No S-9 Microsomes added
9AA = 9-Aminoacridine

SAMPLE FORM

STRAIN: TA 98

DATE: ____________________

(3) POSITIVE CONTROLS - 3x

(1) 2AN (5) + AM
(2) 2AN (1) + AM
(3) 2AN (5) + NM
(4) 2NF (10) + NM
(5) 2NF (5) + NM
(6) DMSO + AM
(7) DMSO + NM
(8) AM
(9) NM
(10) TA 98
(11) ______________
(12) ______________
(13) ______________
(14) ______________
(15) ______________
(16) ______________
(17) ______________
(18) ______________
(19) ______________
(20) ______________

---

(4) STERILITY - 2x

(1) AM Mix
(2) NM Mix
(3) DMSO
(4) 2AN (5)
(5) 2NF (10)
(6) ______________
(7) ______________
(8) ______________
(9) ______________

SA = Sodium Azide
2AN = 2-Anthramine
AM = S-9 Microsomes added
2NF = 2-Nitrofluorene
NM = No S-9 Microsomes added
9AA = 9-Aminoacridine

SAMPLE FORM

STRAIN: TA 98

DATE: ____________________

(3) POSITIVE CONTROLS - 3x

(1) 2AN (5) + AM
(2) 2AN (1) + AM
(3) 2AN (5) + NM
(4) 2NF (10) + NM
(5) 2NF (5) + NM
(6) DMSO + AM
(7) DMSO + NM
(8) AM
(9) NM
(10) TA 98
(11) ______________
(12) ______________
(13) ______________
(14) ______________
(15) ______________
(16) ______________
(17) ______________
(18) ______________
(19) ______________
(20) ______________

---

(4) STERILITY - 2x

(1) AM Mix
(2) NM Mix
(3) DMSO
(4) 2AN (5)
(5) 2NF (10)
(6) ______________
(7) ______________
(8) ______________
(9) ______________

SA = Sodium Azide
2AN = 2-Anthramine
AM = S-9 Microsomes added
2NF = 2-Nitrofluorene
NM = No S-9 Microsomes added
9AA = 9-Aminoacridine

S-9 Preparation Checklist

Day Before

1. Make sure rat food has been removed.

2. Sterilize -

   Scissors and forceps
   Pestles and tubes
   (4-6) Graduated cylinders (100 ml)
   (8-10) Beakers (500 ml)
   Disposable pipets (10 & 25 ml)
   Centrifuge tubes (6-8/batch)
   Kimwipes
   1.15% KCl
   Q-Tips
   Large mouth jars (10/batch)

3. Make nutrient agar plates - need 10.

Harvest Day

1. Need -

   Guillotine
   Paper towels
   Acetone
   Dry ice
   Plastic bags (large and small)
   Cryotubes
   Motor stand + HEX key
   Alcohol
   Medium and large gloves

2. Put note on centrifuge to reserve the appropriate times (10:00 a.m. to 12:00 p.m. and 2:00 p.m. to 4:00 p.m.).

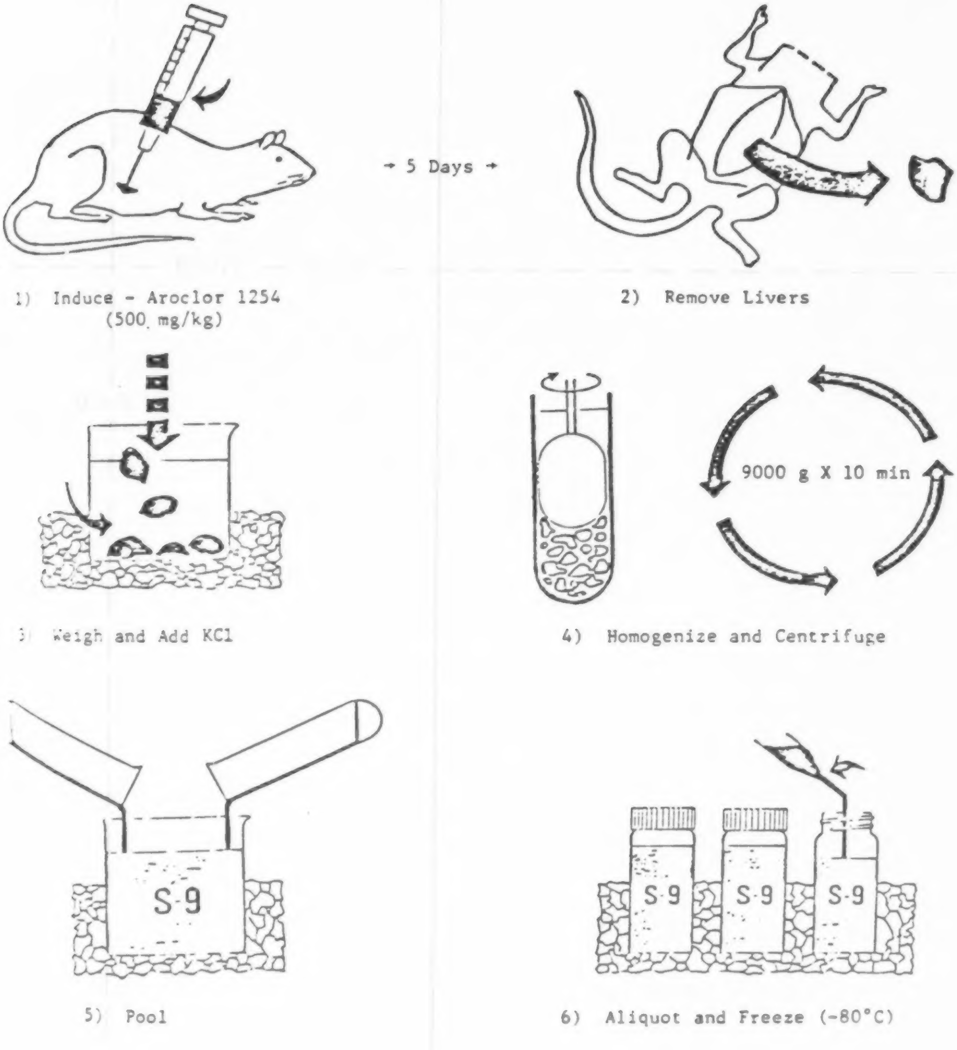
3. Weigh 4-6 sterile beakers.

4. Make certain to keep the bodies separate from other refuse. Bodies are double bagged, put in cold room in animal colony and burned. Refuse is double bagged and put in cold room in Bldg. 3 and buried.

PREPARATION OF S-9 FROM RAT LIVER MICROSOMES (1975)

The procedure of Ames et al., (1975) is used. Male Charles River rats, strain SD (200 g +/-20 g) serve as the source of liver material. The animals are housed one week before initiation of induction in suspended cages. Food is removed from the animal 12 hr before sacrifice. Water is given *ad libitum*. Induced rat liver (minimum of 20 rats) is obtained from rats injected intraperitoneally on day one with 500 mg/kg of Aroclor 1254 in Mazola® Corn Oil (0.5 ml of a 200 mg/ml for a 200 g rat). On day five, the animals are sacrificed with a guillotine. Liver is removed and placed in a sterile jar. Using sterile technique, livers are immersed in cold, sterile 0.15 M KCl, washed two times, and blotted dry with sterile paper (Kimwipes). The livers are placed in a preweighed beaker, weighed, minced, and brought to 200 mg/ml with 0.15 M KCl (3 ml/gm liver). The liver is homogenized (on ice) with four strokes of a cold Potter-Elvehjem apparatus with a Teflon pestle. The homogenate is centrifuged for ten minutes at 10,000 rpm using the Sorvall SS-34 rotor (9,000 g). The liver supernatant is poured in a sterile beaker and aliquoted. The stock solution is checked for sterility, quick-frozen in small aliquots (2-5 ml), in crytotubes and stored for a maximum of two months at -80°C (Revco Ultracold Freezer). For the assay, an aliquot of stock solution is slow-thawed and added to AM mix. Each batch is checked against known positive controls using the plate incorporation assay.

PREPARATION of S-9



1) Induce - Aroclor 1254 (500 mg/kg)

2) Remove Livers

3) Weigh and Add KCl

4) Homogenize and Centrifuge

5) Pool

6) Aliquot and Freeze (-80°C)

APPENDIX W

Laboratory: Southwest Foundation for Research and Education
P.O. Box 28147
San Antonio, TX 78284

Submitter: Dr. Nathan D. Greene
(512)674-1410

Date of submission: February 25, 1981

Omissions: None

SOUTHWEST FOUNDATION FOR RESEARCH AND EDUCATION
P.O. BOX 28147 W. LOOP 410 AT MILITARY DR. SAN ANTONIO, TEXAS 78284 TELEPHONE (512) 674-1410

March 20, 1981

Stephanie Toney
USEPA, HERL, GTD, GBB (MD-68)
Research Triangle Park, NC 27711

Dear Stephanie:

Sincerely regret the delay in remitting our protocol for the Ames Salmonella bioassay. During the past months, we have been actively performing bioassays as well as documenting and submitting new proposals.

I hope our protocol is incorporated in Dr. Claxton's compilation of Ames protocols. I would like to receive a final copy of the compilation and if I can be of any other assistance, do not hesitate to let me know.

Sincerely yours,

Nathan D Greene

Nathan D. Greene, Ph.D.
Southwest Foundation for Research
and Education

SF

R&E

Performance of Ames Salmonella typhimurium Mammalian/Microsome Histidine Reversion Assay as Conducted at Southwest Foundation for Research and Education in San Antonio, Texas

March 1981

SOUTHWEST FOUNDATION
for RESEARCH and EDUCATION

P. O. Box 28147 (W. Loop 410 at Military Drive) • San Antonio, Texas 78284
Bexar County • 21st Congressional District

Performance of Ames Salmonella typhimurium mammalian/microsome histidine reversion assay as conducted at Southwest Foundation for Research and Education in San Antonio, Texas

Performance of Ames Salmonella typhimurium mammalian/microsome histidine reversion assay as conducted at Southwest Foundation for Research and Education in San Antonio, Texas

I. INTRODUCTION

The Ames *Salmonella typhimurium* mammalian/microsome histidine reversion assay is being performed on a regular basis at the Southwest Foundation for Research and Education in San Antonio, Texas. The standard bacterial tester strains we are currently using were obtained by the courtesy of Dr. Bruce N. Ames from the University of California at Berkeley. These tester strains are monitored regularly using diagnostic procedures established in Ames' methods paper to insure the stability and the integrity of each individual tester strain. For each sample tested, we employ tester strains TA 1535, TA 1537, TA 1538, TA 98, and TA 100, which are all histidine dependent cells by virtue of mutations with the histidine functional genetic unit. When these histidine dependent cells are grown on minimal medium agar plates containing a limited amount of histidine, only those cells that revert to histidine independence are able to form colonies. The trace amount of histidine allows all the bacteria plated to undergo a few divisions, which is essential for mutagenesis to occur. It is these histidine independent revertants that are scored as colonies against a slight background growth consisting of histidine requiring cells that have depleted the histidine present within the minimal medium.

In addition of mutations in the histidine functional genetic unit, all the tester strains have a defective lipopolysaccharide coat, which allows large molecules to permeate the bacterial wall, thus increasing bacterial sensitivity to mutagenic aromatic compounds. Furthermore, a U.V. mutation decreases bacteria-damaged DNA repair, thus increasing bacterial sensitivity to additional mutagenic agents. TA 1535 and its plasmid-containing counterpart, TA 100, detect base pair substitutions while TA 1537 and TA 1538 with its plasmid-containing counterpart, TA 98, respond to frameshift mutagens. The plasmids present in TA 98 and TA 100 are believed to cause an increase in error-prone DNA repair which leads to many more mutations. Thus, the five tester strains in tandem provide a very sensitive method for the detection of potential mutagenic environmental samples.

II. METHODS

A. Assay Preparations.

1. Preparation of Minimal Medium. Vogel-Bonner (VB) medium is prepared in three steps. (i) A 50X concentrated VB salt solution is made by first adding and dissolving 10 g of magnesium sulfate ($MgSO_4.7H_2O$) in 670 ml warm (45°C) glass distilled water. One hundred grams of citric acid monohydrate are then added and dissolved before the addition of 500 g potassium phosphate dibasic. Lastly, 175 g sodium ammonium phosphate ($NaHNH_4PO_4.{}^4H_2O$) is added and dissolved prior to aliquoting and autoclaving. Special attention is given to the requirement that each chemical is added and dissolved successively. The final salt 50X concentrate is then aliquoted into 40 and 80 ml portions into storage bottles, which are loosely capped and autoclaved for 15 min at 250°F at 15 p.s.i. (ii) The second step in medium preparation involves making a 20% glucose solution. Two hundred grams of dextrose is dissolved in warm (45°C) glass distilled water and q.s. to 1 liter. One-hundred milliliter aliquots are transferred into storage

bottles which are loosely capped and autoclaved as above. (iii) In the third step, 30 g of Difco Agar are dissolved in 1800 ml glass distilled water and autoclaved. The sterile agar is then allowed to cool to 70°C in a constant temperature bath, and 40 ml of the 50X salt solution and 100 ml of the 20% glucose solution are added. The final medium is then thoroughly mixed and poured (25 ml) into sterile Falcon petri dishes (100 x 15 mm) and allowed to gel at room temperature on a level surface.

2. Preparation of Top Agar. The top agar is prepared by adding 6 g Bacto-agar and 5 g NaCl to 1 liter of distilled water in a 2-liter flask. The final agar concentration is 0.6% and the final NaCl concentration is 0.5%. The contents of the flask are brought to boiling on a heated magnetic stirrer. Aliquots of 100 ml are then transferred to storage bottles and loosely capped, followed by autoclaving as above. The caps are tightened upon removal of the bottles from the autoclave. The bottles are allowed to remain at room temperature until the agar gels, and are then stored at room temperature until needed. For each assay, bottles containing the required amount of top agar are reheated in the autoclave for 6 min at the temperature and pressure described above. The bottles are then placed in a 45°C water bath and allowed to temperature equilibrate for about 2 hr. Each 100 ml of melted top agar is then supplemented with 10 ml of a sterile 0.5 mM L-histidine HCl-0.5 mM biotin solution and gently mixed. Two ml aliquots of the supplemented top agar are then distributed into sterile Kim-capped test tubes (13 x 100 mm) and maintained at 45°C in a Lab-Line multiblock heater for use in the assay.

3. Preparation of Chemicals to be Assayed. All chemicals, whether known or unknown, are weighed in a vented radiological glove box (Labconco) housing a

four place Mettler balance. The chemicals are weighed in individual sterile silicone rubber stoppered test tubes (13 x 100 mm). Tubes containing the weighed chemicals are transferred from the glove box into the laminar flow hood (Baker Biogard) and placed into solution with an appropriate amount of a suitable sterile solvent, usually dimethylsulfoxide (DMSO). The stock chemicals are further diluted into sterile stoppered tubes containing the appropriate volumes of sterile solvent. Liquid chemicals to be tested are diluted into appropriate solvents under similar conditions and precautions. Dilutions of all materials to be assayed are arranged so that the amount of chemical to be added per plate will be contained in 0.1 ml. Sufficient material is placed in solution so that triplicate plates can be assayed at each concentration, with and without metabolic activation.

4. Preparation of S-9 Mix (Metabolic Activation System). For preparation of the liver S-9 fraction, we routinely use Sprague-Dawley male rats weighing approximately 200 g.

Animals are given a single i.p. injection of Aroclor 1254 diluted in corn oil to a concentration of 200 mg/ml at a dosage of 500 mg/kg body weight 5 days prior to sacrifice. Twelve hours prior to sacrifice, food is removed and on the fifth day, the animals are stunned by a blow to the head and decapitated. Using sterile techniques, the liver is removed and weighed in a previously tared beaker containing cold 0.15 M KCl. The livers are subsequently washed once in one volume of cold, sterile 0.15 M KCl per weight of wet tissue, and then placed in three volumes cold sterile 0.15 M KCl per weight tissue. Using sterile techniques, the liver is minced and then transferred into a Potter-Elvejhem tissue grinder and homogenized with a Teflon pestle. The homogenate is centrifuged in sterile capped tubes at 9,000 g for 10 min in a Sorval RC refrigerated centrifuge at 4°C. The supernatant (S-9) fraction is aliquoted in 3- and 10-ml volumes into sterile capped plastic test tubes and immediately frozen in dry ice. The tubes are stored until needed in a Revco freezer at -80°C. Protein concentration of each batch of S-9 is determined (Biorad Protein Assay), and is checked to ensure that enzyme activity is within the desired range. The S-9 preparations are used within 3 months of preparation.

5. Preparation of Cofactors. The cofactor solution consists of D-glucose-6-phosphate, NADP monosodium salt, magnesium chloride, potassium chloride, monobasic and dibasic sodium phosphate. For each 100 ml of cofactor solution, we use 0.157 g D-glucose-6-PO_4, 0.350 g NADP, 0.180 g $MgCl_2$, 0.273 g KCl, 1.278 g dibasic sodium phosphate and 0.278 g monobasic sodium phosphate and q.s. to 100 ml in glass distilled water. When all the chemicals are in solution, the solution is sterile filtered with a disposable 100 ml Nalgene filter unit. The sterile cofactor solution is then dispensed into sterile screw capped tissue culture tubes in 9 ml aliquots, quickly frozen, and stored at -80°C. Upon determination of the amount of S-9 mix needed for a given assay, appropriate amounts of S-9 homogenate and cofactor solutions are thawed and mixed at a ratio of 1 ml S-9 homogenate to 9 ml of cofactor solution. Therefore, using the 1 to 9 ratio, 1 ml of S-9 mix contains the following:

G-6-PO_4	1.413 mg
NADP	3.15 mg
$MgCl_2$	1.62 mg
KCl	2.457 mg
$Na_2HPO_4 \cdot 7H_2O$	11.502 mg
$NaH_2PO_4 \cdot H_2O$	2.502 mg

6. Preparation of the Tester Strains. The procedures described here apply to all five tester strains which are commonly used in our laboratory: TA1535, TA1537, TA1538, TA98 and TA100. Frozen aliquots of cultures of each strain are removed from the -80°C freezer and inoculated into individual Kim-capped test tubes (18 x 150 mm), each containing a volume of oxoid nutrient broth No. 2 appropriate for the extent of the assay to be conducted, but not less than 5 ml. Inoculation is accomplished by rimming the surface of the frozen culture with a sterile applicator stick, and transferring the culture into the nutrient broth tube. The frozen culture aliquots are used only once and discarded to avoid potential deleterious effects of partial surface thawing and subsequent re-freezing. In our laboratory, the tester strains are inoculated into the nutrient broth at 4:30 p.m. and incubated overnight at 37°C in a Forma Reach-In incubator. At 8:30 a.m. the following morning, when the assay is to be conducted, the bacterial cultures are removed from the incubator and maintained at 4°C in wet ice while the other necessary preparations for the assay are completed.

B. The Plate Incorporated Assay.

While the tester strains are maintained in an ice bath, the top agar is melted, supplemented with 0.5 mM histidine and 0.5 mM biotin, and then distributed in 2-ml aliquots into 13 x 100 mm test tubes which are placed in a multiblock heater maintaining a temperature of 45°C. The S-9 mix is prepared as described above and chilled on wet ice. The chemicals are weighed, placed in solution and diluted to the appropriate concentrations for the assay, as described above. The minimal medium plates are properly labeled in triplicate and transferred into the laminar flow hood in groups of 15. The refrigerated bacterial cultures and the S-9 mix, still maintained on ice, are brought into the hood. The usual procedure is first to inoculate each 13 x 100 mm test tube containing top agar with 0.1 ml of the appropriate bacterial culture, followed by 0.1 ml of test chemical, and then add 0.5 ml of S-9 mix. Each plate therefore receives 50 µl of the S-9 homogenate and 450 µl cofactor solution. The test tube is then removed from the block heater and vortex mixed for approximately 2 sec; the tube contents are poured into the appropriate prelabeled plate, which is gently tilted to evenly spread the top agar over the entire surface of the minimal medium. The plates are then allowed to remain in the hood at room temperature for gelling of the top agar while other plates are poured in the same manner. When the plates have been allowed to gel for at least 30 min, they are inverted in groups of 15, removed from the hood, and placed inside the vented Forma Reach-In incubator. After an incubation period of 72 hr, the plates are removed from the incubator and the number of revertant colonies on each plate determined.

Adhering to the standard plate incorporated assay as described by Ames and following suggestions prescribed by EPA, each sample is tested at seven different chemical concentrations on all five indicator strains with and without a metabolic activation system. Each sample tested is also coupled with its own set of negative and positive controls for all tester strains. Controls include: (1) media control, (2) solvent controls, (3) S-9 mix control, (4) positive controls using diagnostic mutagens in the absence of a metabolic activation system, and (5) positive controls, again using a diagnostic mutagen, but in the presence of an S-9 mix.

We routinely use an electronic colony counter (New Brunswick Biotran II), which is regularly calibrated against manual counts over the range of colony counts expected in routine assays. Bacterial lawns are examined for evidence of normal growth with the aid of a Bausch and Lomb dissecting microscope.

C. Assessment of Genotypic Stability of Tester Strains.

We regularly perform diagnostic tests to confirm the stability and integrity of the tester strains. For this purpose, samples of the five strains are inoculated into 5 ml of nutrient broth and allowed to grow for approximately 16 hr at 37°C. At the end of the incubation period, the bacteria are streaked on nutrient agar plates and incubated for an additional 24 hr for the purpose of recovering single cell isolates of each of the strains. Three isolates of each tester strain are obtained as a minimum, inoculated into culture tubes with 5 ml nutrient broth, and again incubated overnight. This provides three samples per strain of each of the five strains for sensitivity testing.

In assaying for crystal violet, ampicillin, and UV sensitivity, we use nutrient agar plates. An aliquot (0.1 ml) of each culture is added to 2.0 ml nutrient top agar, mixed and poured onto a prelabeled nutrient agar plate. With sterile forceps, a crystal violet disc (0.25 inch with 10 μl of a 1 mg/ml crystal violet solution) is placed in the center of each plate. The plates are then incubated overnight, and the zone of inhibition around the disc determined. A strain is acceptable (i.e., has a good rfa) if the zone of inhibition is 14 mm or greater.

To determine ampicillin resistance, a sterile solution of 8 mg/ml ampicillin in 0.02 N NaOH is prepared and 0.1 ml of the solution spread to dryness on two nutrient agar plates. Two plates without ampicillin serve as the control for the test, as well as the control for the UV sensitivity test below. Each of the four plates is divided into eight sections, and the three samples of each of the five strains are streaked on ampicillin plus and ampicillin minus sections. The plates are then incubated overnight at 37°C, and the ability to grow in the presence of ampicillin determined.

For the UV sensitivity test, two nutrient agar plates are sectioned as above into eight areas. All 15 samples are streaked on the sectioned plates and placed under a UV lamp for 8 sec. The UV lamp is warmed and functioning for a minimum of 15 min before exposing the plates. The UV lamp is placed approximately 33 cm from the top of the plates and the covers of the plates are removed prior to exposure. After exposure, the plates are incubated overnight at 37°C, and are then examined for evidence of sensitivity to UV by the presence or absence of growth.

In testing for the other diagnostic requirements, such as histidine and biotin requirement, and spontaneous mutation rates, we use minimal medium agar plates. For spontaneous reversion rates, each sample is assayed in triplicate; therefore 45 minimal medium plates plus 45 test tubes (13 x 100) with 2 ml of top agar supplemented with a biotin-histidine solution are used. To 2 ml of top agar is added 0.1 ml of the bacterial culture to be tested. This is mixed by vortexing and is poured onto a properly labeled petri dish with minimal medium. The plates are incubated at 37°C for 48 hr, after which time the number of revertant colonies are counted. Acceptable levels of spontaneous reversion in our laboratory are as follows: TA1535 - 10-35; TA1537 - 3-15; TA1538 - 15-35; TA98 - 20-50; and TA100 - 75-150. When the number of spontaneous revertants per plate fails to fall within the acceptable range, new stocks are prepared by cloning and subsequent selection of clones with the desired activity. The new stocks are aliquoted and stored frozen at -80°C.

In testing for histidine and biotin requirement, we use stock solutions of sterile histidine (1 g/100 ml glass distilled water) and sterile biotin (10 mg/100 ml water). The tests require a total of six minimal medium agar plates. On two plates is added 0.1 ml of the histidine solution. Using an L-shaped glass rod, the solution is spread throughout the surface of the plates until dry. To the second pair of plates is added 0.1 ml of the biotin solution which is similarly spread. To the third pair of plates is first added 0.1 ml of the histidine solution followed by 0.1 ml of the biotin solution which are, in sequence, spread until dry. The plates are divided into eight sections, and are streaked with the cultures as described above. They are subsequently incubated overnight at 37°C and then examined for the presence of normal growth.

For each of the 15 isolates which had been plated and tested for crystal violet, UV, ampicillin, histidine, and biotin sensitivities, and for the number of spontaneous reversion rate, a data chart is constructed. The zones of inhibition around the crystal violet disk are measured and the value entered into the data chart. All tester strains should be sensitive to UV, and they should be able to grow in the combination of histidine and biotin. Tester strains TA 1535, TA 1537, and TA 1538 should be sensitive to ampicillin, and TA 98 and TA 100 should be resistant to ampicillin. From the information in the chart, the single cell isolates which are most representative of published, and our own historical, acceptable values are used to prepare stock cultures for storage. Isolates are inoculated into culture tubes with 5 ml nutrient broth and are incubated overnight at 37°C. After 16 hr growth, the tester strain cultures are diluted with DMSO, following recommendation of the Ames group, and quickly frozen in dry ice and stored in a Revco freezer at -80°C.

D. Assay Sequence.

An initial range finding experiment is conducted with each compound prior to initiation of definitive assays. In the preliminary study, solubility of the compound in solvents compatible with the Salmonella assay system, beginning with water and progressing to DMSO, dimethylformamide, or other solvents recommended by the Ames group is determined. Samples assessed for solubility up to 50 mg/ml (i.e., 10 mg/200 µl to be used in each plate), and for lack of precipitation upon addition to top agar solutions at concentrations to be used in the assay.

Upon determining the limits of solubility, a range finding toxicity study is conducted, covering a range from 1 µl/plate up to 10,000 µl/plate (or the solubility limiting concentration) in seven steps. Each concentration is tested without replicates in each of two tester strains, TA1538 and TA100, both with and without metabolic activation with rat liver S-9 preparation.

Using the information generated in the preliminary study, the range of concentrations to be included in the final assay will be determined. This consists of the lowest concentration showing toxicity to either of the tester organisms, as indicated by abnormal or absent growth of the lawn, or the lowest concentration at which precipitation occurred or at which the compound was insoluble, or 10 mg/plate. The assay is conducted as described previously using five tester strains in triplicate, with seven concentrations of the test compound, extending downward to 1 µl/plate, with and without metabolic activation with rat liver S-9 and hamster liver S-9. The assay is then repeated, using the same concentrations as were used in the initial assay.

III. FACILITIES AND EQUIPMENT

All facilities and equipment necessary to conduct Ames bioassays are available within the Immunotoxicology and Environmental Mutagenesis Laboratory at Southwest Foundation for Research and Education (SFRE). The Bacterial Mutagenesis Laboratory is a separate self-contained unit occupying approximately 450 square feet. A separate, inner room within the laboratory houses a Labconco glove box in which test chemicals are weighed and aliquoted. The glove box is vented to outside air through HEPA filters and is equipped for power ventilation. A Baker Biogard 6 foot laminar flow hood, also vented to outside air through HEPA filters, is used for seeding plates, incorporation of chemicals into reaction mixtures and other manipulations requiring maintenance of sterility. Plates are incubated in a Forma Reach-In incubator located adjacent to the hood, and has the capacity to house the plates for up to four complete assays simultaneously. The outside area within the laboratory comprises work counters and tables for media preparation, examination and counting of plates, chemical preparation, etc. A chemical fume hood is available for working with solvents. The BioTran II (New Brunswick Scientific) electronic colony counter is located within the outer work area, on a work bench also equipped with a Bausch and Lomb dissecting microscope used for examination of the plates for evidence of microbial growth or lack of growth. An adjacent media preparation room contains an autoclave and other equipment necessary for media preparation, pouring of plates, etc.

Animal facilities necessary for maintaining the rats and hamsters required for preparation of induced liver enzyme preparations are available. These facilities meet the requirements for working with hazardous materials, and are currently used for toxicological studies with various toxic agents. The animal facilities at SFRE are operated within the department of Laboratory Animal Medicine, and are supported by a complete staff of veterinary and support personnel especially trained in animal care. The facilities are accredited under the rules and regulations of the American Association for Laboratory Animal Science and are licensed by the United States Department of Agriculture according to Public Law 91-579.

IV. SAFETY AND HEALTH PRECAUTIONS

All activities related to working with suspected or known chemical carcinogens are conducted in facilities designed specifically for that purpose, i.e., controlled access areas with non-recirculating air handling capabilities and equipped with all proper protective devices. Upon receipt, containers are placed in a lockable storage cabinet, located within the biohazard laboratory, which is ventilated through HEPA filters to outside air.

In preparation for assays, chemicals are weighed in a radiological glove box, and the aliquots to be assayed are transferred in sealed containers to a laminar flow hood where the actual assay set up is performed. Solvents, media, etc., are handled in a chemical fume hood and are subsequently transferred in sealed containers to the laminar flow hood for use.

During all phases of the assay, proper protective clothing is worn. This consists of a fully closed long laboratory coat, face mask, and disposable gloves.

All waste materials generated during the course of an assay are collected in waste containers designed for this purpose, i.e., the "Terminal Biohazard System" (Whitney Corp.). The packages are sealed and all materials, including the plates at the end of the assay, are disposed of by incineration. The incineration system at SFRE is specifically designed for dealing with highly infectious and otherwise hazardous waste materials. Our laboratories comply with all applicable statutes regarding occupational safety and health, transportation and handling of hazardous materials, and environmental protection. Facilities and laboratory practices are in agreement with guidelines established by appropriate agencies, i.e., as described by Barbeito (1979) and Sontag, Page and Saffiotti (1976). All laboratories at SFRE adhere to the rules and regulations regarding good laboratory practice as established by the U.S. Food and Drug Administration.

APPENDIX X

Laboratory: SRI International
333 Ravenswood Ave.
Menlo Park, CA 94025

Submitter: Dr. David C.L. Jones

Date of submission: December 18, 1980

Omissions: None

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
HEALTH EFFECTS RESEARCH LABORATORY
RESEARCH TRIANGLE PARK
NORTH CAROLINA 27711

November 26, 1980

Dr. David C.L. Jones
SRI International
333 Ravenswood Avenue
Menlo Park, CA 94025

Dear Dr. Jones:

Since you head a well-known laboratory in the Ames Salmonella assay I would like to request a current Ames Test Protocol from you. I am working for Dr. Larry Claxton in preparing a compilation of Ames test protocols for the Environmental Protection Agency. This compilation will serve several purposes:

(1) A reference for workers within the genetic toxicology field,

(2) a starting point for the creation of reference protocols for EPA laboratories, industrial laboratories, and others who have a need for precise Ames test protocols, and

(3) as a guide to understanding variations in Ames test results.

Your protocol is needed in order to document differences between protocols in this compilation.

A final copy of the compilation will be sent to you if you would like one.

Please address the protocol to:

Stephanie Toney
USEPA, HERL, GTD, GBB (MD-68)
Research Triangle Park, NC 27711

Thank you for your prompt consideration.

Sincerely yours,

Stephanie Toney

Stephanie Toney
Genetic Bioassay Branch (MD 68)

Protocol sent 12/18/80

B. *SALMONELLA TYPHIMURIUM* PLATE INCORPORATION ASSAY (AMES TEST)

1. Background

a. Genetic Basis of Test

The *Salmonella typhimurium* strains used at SRI are all histidine auxotrophs by virtue of mutations in the histidine operon. When these histidine-dependent cells are grown on minimal medium agar plates containing a trace of histidine, only those cells that revert to histidine independence (*his*$^+$) are able to form colonies. The small amount of histidine allows all the plated bacteria to undergo a few divisions; in many cases, this growth is essential for mutagenesis to occur. The *his*$^+$ revertants are easily visible as colonies against the slight background growth. The spontaneous mutation frequency of each strain is relatively constant (McCann et al., 1975a; Mortelmans, 1978), but when a mutagen is added to the agar, the mutation frequency is increased, usually in a dose-related manner.

b. Source of Strains

The tester strains used at SRI are TA1535, TA1537, TA1538, TA98, and TA100. They were obtained from Dr. Bruce Ames of the University of California at Berkeley (Ames et al., 1975). In addition to having mutations in the histidine operon, all the indicator strains have a mutation (*rfa*) that leads to a defective lipopolysaccharide coat; they also have a deletion that covers genes involved in the synthesis of the vitamin biotin (*bio*) and in the repair of ultraviolet (uv)-induced DNA damage (*uvrB*). The *rfa* mutation makes the strains more permeable to many large molecules, thereby increasing the mutagenic effect of these molecules. The *uvrB* mutation causes decreased repair of some types of chemically or physically damaged DNA and thereby enhances the strains' sensitivity to some mutagenic agents. Strain TA1535 is reverted to *his*$^+$ by many mutagens that cause base-pair substitutions. TA100 is derived from TA1535 by the introduction of the resistance transfer factor, plasmid pKM101. This plasmid is believed to cause an increase in error-prone DNA repair that leads to many more mutations for a given dose of most mutagens (McCann et al., 1975b). In addition, plasmid pKM101 confers resistance to the antibiotic ampicillin, which is a convenient marker to detect the presence of the plasmid in the cell (Mortelmans and Stocker, 1979). The presence of this plasmid also makes strain TA100 sensitive to some frameshift mutagens [e.g., ICR-191, benzo(a)pyrene, aflatoxin B_1, and 7, 12-dimethylbenz(a)anthracene]. Strains TA1537 and TA1538 are reverted by many frameshift mutagens. Strain TA98 is derived from TA1538 by the addition of the plasmid pKM101, which makes it more sensitive to some mutagenic agents (Ames et al., 1975).

All indicator strains are kept at 4°C on minimal agar plates supplemented with an excess of biotin and histidine. The plates with the plasmid-carrying strains also contain ampicillin (25 μg/ml) to ensure stable maintenance of the plasmid pKM101. New stock culture plates are made every 4 to 6 weeks from single colony isolates that have been checked for their genotypic characteristics (his, rfa, uvrB, bio) and for the presence of the plasmid. For each experiment, an inoculum from the stock culture plates is grown overnight at 37°C in nutrient broth (Oxoid, CM67).

2. Method

The specific test method has been described in detail (Ames et al., 1975; Mortelmans, 1978).

a. General

To a sterile 13 × 100 mm test tube placed in a 43°C heating block, we will add in the following order:

(1) 2.00 ml of 0.6% agar*

(2) 0.05 ml of indicator organisms

(3) 0.50 ml of metabolic activation mixture (if appropriate)

(4) 0.05 ml of a solution of the test chemical.

This mixture will be stirred gently and then poured onto minimal agar plates.† After the top agar has set, the plates will be incubated at 37°C for 2 days. The number of his$^+$ revertant colonies will then be counted and recorded.

b. Solvent

The solvent of choice will be distilled water or 0.067 M phosphate buffer (pH 7.4). Chemicals that do not dissolve in an aqueous solvent will be dissolved in dimethylsulfoxide (DMSO). Test chemicals will be prepared on the day they are tested.

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[*] The 0.6% agar contains 0.05 mM histidine, 0.05 mM biotin, and 0.6% NaCl.

[†] Minimal agar plates consist of, per liter: 15 g of agar, 10 g of glucose, 0.2 g of $MgSO_4 \cdot 7H_2O$, 2 g of citric acid monohydrate, 10 g of K_2HPO_4, and 3.5 g of $NaNH_4HPO_4 \cdot 4H_2O$.

c. Compound Dilution

Serial dilutions will be made from initial stock.

d. Controls

Concurrent negative, solvent, and positive (known mutagens) controls will also be run. The following positive control chemicals will be used:

- Sodium azide for the base-pair substitution mutants TA1535 and TA100.
- 9-Aminoacridine for the frameshift mutant TA1537.
- 2-Nitrofluorene for the frameshift mutants TA1538 and TA98.
- 2-Aminoanthracene for all tester strains, in the presence of metabolic activation.

The negative control preparation will contain neither test compound nor solvent.

e. Metabolic Activation System

Some carcinogenic chemicals (e.g., of the aromatic amine type or the polycyclic hydrocarbon type) are inactive unless they are metabolized to active forms. In animals and man, an enzyme system in the liver or other organs (e.g., lung or kidney) is capable of metabolizing a large number of these chemicals to carcinogens. Some of these intermediate metabolites are very potent mutagens in many in vitro short-term tests. Ames et al. (1975) described the liver metabolic activation system that we use. In brief, adult male Fischer 344 rats weighing 180-250 g will be given a single 500 mg/kg intraperitoneal injection of Aroclor 1254 (a mixture of polychlorinated biphenyls). This treatment enhances the synthesis of enzymes involved in the metabolic conversion of chemicals. Four days after the injection, the animals' food will be removed but drinking water will be provided ad libitum. On the fifth day, the animals will be killed and the liver homogenate prepared as follows.

The livers will be removed aseptically and placed in a preweighed sterile glass beaker. The organ weight will be determined, and all subsequent operations will be conducted in an ice bath. The livers will be washed with an equal volume of cold, sterile 0.15 M KCl (1 ml/g of wet organ), minced with sterile surgical scissors in three volumes of 0.15 M KCl, and homogenized with a Potter-Elvehjem apparatus. The homogenate will be centrifuged for 10 minutes at 9000 × g, and the supernatant, referred to as the S-9 fraction will be frozen quickly in dry ice and stored at -80°C. Lowry protein (Lowry et al., 1951), aryl hydrocarbon hydroxylase (Van Cantfort et al., 1977), and sterility determinations will be made on each new batch of S-9.

The metabolic activation mixture for each experiment will consist of, for 10 ml:

- 1.00 ml of S-9 fraction
- 0.20 ml of $MgCl_2$ (0.4 M) and KCl (1.65 M)
- 0.05 ml of glucose-6-phosphate (1 M)
- 0.40 ml of NADP (0.1 M)
- 5.00 ml of sodium phosphate buffer (0.2 M, pH 7.4)
- 3.35 ml of H_2O.

f. Preliminary Procedure

A preliminary range-finding test will be run with strain TA100 on all chemicals to determine a suitable nontoxic range of dose levels. Each chemical will be tested at a minimum of six dose levels, with the highest nontoxic dose level tested being 10 mg/plate unless specific solubility dictates a lower upper limit. The dose levels tested will be reduced appropriately for toxic chemicals. Ideally, at least four nontoxic dose levels will be tested.

g. Procedure for Final Testing

Compounds will be tested at six dose levels covering a wide range of concentrations in the presence and in the absence of a mammalian liver metabolic activation system. The highest dose used will depend on the solubility and toxicity of the test compound. All assays will be repeated at least once on a separate day.

3. Raw Data Collection

Experimental data for all assays will be recorded on the form shown on the next page and will be placed in laboratory notebooks.

Our electronic colony counters are interfaced with a PDP/11-03 minicomputer. This allows us to enter data automatically into the computer using the computerized result formats, thereby eliminating transcriptional errors. A display screen allows us to monitor the data entry, and a terminal prints out the results in the appropriate format.

4. Data Manipulation and Presentation

A mutagenic response is defined as a dose-related, reproducible increase in the number of histidine-independent colonies per plate. The actual number of colonies observed on the plates will be reported.

IN VITRO ASSAYS
SALMONELLA TYPHIMURIUM

Compound	Metabolic Activation	µg of Compound Added per Plate	Histidine-Positive Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100

Only raw data, no transformed data, will be presented in table form. An experiment will be considered invalid when control values are not within historical limits, or when three or more of the six dose levels selected exhibit toxicity, and will be repeated with appropriately adjusted dose levels.

5. Criteria for Interpretation

Positive. A compound will be considered a mutagen when it produces a reproducible, dose-related increase in the number of revertants in one or more strains. This increase should occur on at least three dose levels.

Negative. A compound will be considered a nonmutagen when no dose-related increase in the number of revertants is observed in at least two independent experiments. The maximum dose level tested for nontoxic compounds should be 10 mg/plate (unless dictated otherwise by solubility problems). For toxic compounds, only the highest dose level tested should show evidence of toxicity.

Inconclusive. When a compound cannot be identified clearly as a mutagen or nonmutagen in the standard plate assay, the results will be classified as inconclusive.

6. Sample Requirement

Approximately 0.5 g of test material is required to perform the Salmonella/microsome assay.

7. Time Requirement

Normal. Six weeks will be required from the time of sample receipt to perform the test and report the results.

Rapid Response. Four weeks will be required to test and report on samples requested to be tested when the Project Officer requests a rapid response.

8. Test Variations—Assays in Desiccators

Highly volatile chemicals are difficult to test in the normal assay procedures because of loss of material during the incubation step. We have developed a special procedure for this. The Salmonella plates will be prepared as described for the assays in agar, but no test chemical will be added. The plates, the lids having been removed, will be placed side by side on a perforated shelf in a 9-liter desiccator. A known volume of the test chemical will be added to a glass petri plate placed in the center of and attached to the bottom of the shelf. A control chemical will be tested similarly in each experiment. The desiccator will be sealed and placed on a magnetic stir-plate in a room maintained at 37°C. A magnetic stirrer with vanes, placed in the base of each desiccator, ensures adequate dispersion of the chemical. After incubation for 8 hours, the plates will be removed from the desiccators, their lids will be replaced, and they will be incubated at 37°C for an additional 42 hours. The number of $\underline{his}^{+}$ revertants will be counted and recorded.

9. References

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Mortelmans, K. E., and B.A.D. Stocker. 1979. Segregation of the mutator property of plasmid R46 from its ultraviolet-protecting property. Mol. Gen. Genet. 167, 317-327.

Mortelmans, K. E. 1978. Salmonella/mammalian-microsome test to detect chemical mutagens. J. Food Prot. 41, 989-995.

Van Cantfort, J., J. DeGraeve, and J. E. Gielen. 1977. Radioactive assay for aryl hydrocarbon hydroxylase. Improved method and biological importance. Biochem. Biophys. Res.

APPENDIX Y

Laboratory: ES Unilab Research, Inc.
2800 Seventh St.
Berkeley, CA 94710

Submitter: Melanie Baltizore
Laboratory Manager
(415)548-8440

Date of submission: December 5, 1980

Omissions: Mutagen Test Screen
Preincubation Test

ES UNILAB RESEARCH, INC.
2800 SEVENTH STREET, BERKELEY, CALIFORNIA 94710 (415) 548-8440

December 5, 1980

Stephanie Toney
USEPA, HERL, GTD, GBB(MD-68)
Research Triangle Park, NC 27711

Dear Ms. Toney:

Thank you for your interest in our Ames Test protocols. Enclosed are copies of three protocols for the Ames test, which we use depending on the needs of our clients.

We appreciate your attempt to standardize this relatively new area in industrial testing.

If you have any questions or comments, please feel free to contact us.

We would appreciate receiving a copy of the compilation when it is completed.

Sincerely,

ES UNILAB RESEARCH

Melanie Baltezore
Laboratory Manager

MB/dh

enc.

TOXICITY DETERMINATIONS – BIOASSAYS – NUTRITIONAL EVALUATIONS
U.S. Food & Drug Administration Testing Requirements

ES UNILAB RESEARCH, INC.

MUTAGEN TEST ASSAY II

The basic procedure for performing the *Salmonella* short-term assay is that described by Ames *et al*. (1) as modified by a Supplement (2) and Workshop reports (5). Our protocol, as described below, includes the preincubation of the potential mutagen at 4 levels using 3 strains in duplicate with known mutagen controls.

Strains

Salmonella typhimurium strains TA1537, TA98 and TA100 are used in assessing mutagenicity of a compound and have been described by Ames *et al*. (1).

Maintenance of Strains

Tester strains are obtained from the Department of Biochemistry, University of California, Berkeley and "Frozen Permanents" are prepared on each strain after the phenotype is confirmed. Strains are inoculated into Oxoid Nutrient Broth No. 2 supplemented with 10 µg/ml histidine and grown overnight with moderate shaking. Spectrophotometric quality DMSO is added (0.07 ml DMSO to 0.8 ml culture) and the cultures are dispersed to labeled vials and transferred to -80°C storage.

The frozen cultures are divided into two batches for use as either "Master Copies" or "Routine Permanents".

A "Master Plate" is prepared from "Routine Permanents" on each strain and is used for the generation of inoculum for routine assays over a two month period of time.

Maintenance of Strains (Cont.)

Master plates are prepared by spreading 0.2 ml of a solution of histidine (0.1 M) and biotin (0.5 mM) onto the surface of a minimal glucose agar plate. For strains containing R factors, 0.1 ml of ampicillin (8 mg/ml) also is added. These plates are prepared 2-3 days before inoculation from a culture obtained by opening and streaking from a Routine Permanent vial. The master plates are enclosed in plastic "Baggies" and kept at 4°C for no longer than 2 months, at which time another set of master plates is prepared.

Phenotype of Strains

The appropriateness of each strain to be used as a "tester strain" is assured by examining the phenotype of that strain. The phenotype of each strain is examined upon receiving the culture (from Berkeley) and each time a new master plate is prepared. If the phenotype of the strain is unsatisfactory for testing purposes, it is restreaked and six colonies are then examined for their phenotypic characteristics. An appropriate colony is then chosen to be the strain for assay purposes.

The following phenotypes are examined:

a) Ampicillin resistant

Since we use the master plate technique for maintaining working stocks and since ampicillin is incorporated into the agar of master plates used to grow strains containing R factors (TA98, TA100), no assay for ampicillin is necessary.

b) Deep Rough (Rfr) phenotype

The presence of the deep rough phenotype is assayed by adding 0.1 ml (10^8 cells) culture to top agar supplemented with 0.1 ml histidine-biotin mixture (0.1 M histidine, 0.5 mM biotin) and poured over minimal glucose

Phenotype of Strains (Cont.)

agar plates. A sterile paper disc containing 0.1 ml of a 1 mg/ml solution of crystal violet is placed in the center of the plate and the plates incubated overnight. A zone of inhibition of approximately 14-15 mm diameter indicates the presence of the deep rough character.

c) Ultraviolet sensitivity

We use the streak plate assay of Greenberg to assess ultraviolet sensitivity. An inoculum of culture is streaked across a plate of Oxoid Nutrient Agar beside a streak of wild-type strain ST2. The plates are divided into 8 sections with marked lines. The plates are covered with a sterile piece of heavy duty aluminum foil which is moved across the sections, except the last section, at 10 second intervals. The plates are exposed to an ultraviolet source from a 30 cm distance. This permits each section to be irradiated by increasing ten second intervals. After 48 hours incubation in the dark, wild-type cells have survivors after 60 second exposure, whereas ultraviolet sensitive cells have scant or no survivors after 10 second exposure.

d) Culture and cell morphology

The appearance of cells grown in Oxoid Nutrient Broth are examined microscopically for any undue clumping while the colonial morphology of cells grown on Oxoid Nutrient Agar are examined microscopically under oblique lighting for homogeneity in colony morphology and size.

e) Spontaneous Mutation Frequency

An overnight shaken broth culture is diluted tenfold in phosphate buffer to measure spontaneous mutants and a 10^{-6} dilution is made (in

Phenotype of Strains (Cont.)

three 10^{-2} steps) to measure viability. Viability is determined by adding 0.1 ml to a tube containing 2 ml soft agar (maintained at 45°C and supplemented with 0.05 mM biotin and 60 µg/ml histidine). The tube is gently swirled and poured over the surface of a minimal agar plate. Duplicates are prepared for all determinations. The preexisting mutant frequency is determined by adding 0.1 ml of 10-fold diluted culture to top agar supplemented with 0.05 mM biotin (no histidine) while the spontaneous plate mutant frequency is determined by adding 0.1 ml of the tenfold diluted culture to top agar containing 0.05 mM biotin and 0.05 mM histidine. The plates are stacked in groups of no more than five in a 37°C incubator and the colonies are counted manually at the end of 72 hours of incubation.

The number of plate mutants is determined by subtracting the preexisting mutants from the value obtained for the spontaneous plate mutants (Table 1). The number of plate mutants is dependent upon the number of generations a strain undergoes under histidine-limiting conditions. Since the histidine concentration is controlled, this value is more reproducible than fluctuations obtained in broth cultures and is thus used to compare the spontaneous mutation frequency. The acceptable median of revertants for each strain is that described by Ames et al. (1) and the acceptable range is that described by duSerres and Shelby (6). Comparisons are made only on results obtained with cell densities between 5×10^7 and 2×10^8 cells/plate.

Induced Mutation Frequency

The induced mutation frequency protocol incorporates the previously described spontaneous mutation frequency protocol and uses the data obtained from it for correcting the data obtained from the measurement of the induced mutation frequency. These are performed together.

This assay is the standard assay and is performed on each strain upon receiving the culture and every time a new master plate is prepared with standard mutagens. It is also the assay for assessing for the mutagenicity of a compound. Therefore, the detailed procedures and materials for the various parts of this assay are described below.

Media

a) Oxoid Nutrient Broth No. 2 is used supplemented with 10 μg/ml L-histidine. Oxoid Nutrient Broth No. 2 supplemented with 1.5% Difco Agar is used as nutrient agar.

b) Minimal glucose agar. Petri dishes contain 30 ml (added as precisely as possible) of minimal glucose agar which contains 1.5% Difco Agar, 2% glucose and Vogel-Bonner medium (final concentrations in g/1:0.2g $MgSO_4 \cdot 7H_2O$; 2.0 g citric acid (hydrated); 10.0 g K_2HPO_4 (anhydrous); 3.5 g $Na(NH_4)\ HPO_4 \cdot 4H_2O$). Agar and salts are autoclaved separately. Glucose is filter sterilized.

c) Soft agar. Soft agar contains 0.6% Difco agar, 0.05% NaCl, supplemented with 0.05 mM biotin and 0.05 mM histidine, and is added as an overlay to the minimal agar in the petri dishes in 2 ml aliquots. It is autoclaved without histidine and biotin and is stored in bottles in volumes of 100 ml at room temperature. Before use, the agar is melted by heating the bottle in a steam bath and 10 ml of a sterile (sterilized by filtration) solution of 0.05 mM

Media (Cont.)

L-histidine 0.05 mM biotin is added to the molten agar and mixed thoroughly. Soft agar supplemented with biotin but no histidine is required to determine the number of pre-existing mutants in the culture, and soft agar supplemented with biotin and 60 μg/ml histidine is required to determine the viable count per plate.

Materials and Sources

Plastic petri dishes are purchased from Plasta Medic. Pepets (Kimex) and pipettor tips (Eppendorf) are supplied by VWR. Salts in the minimal glucose agar are reagant grade and are obtained from Mallinchrodt. The nutrient broth is purchased from Oxoid, while Difco serves as the source of agar. Sigma supplies the NADP, 2 anthramine, 9 aminoacridine, dimethyl nitrosamine and αbenzopyrene. Glucose, Histidine-HCl, Biotin and glucose-6-phosphate are purchased from United States Biochemicals. The activation system is supplied by Meloy Laboratories in the form of a S-9 preparation of "mutazyme", which is the dehydrated S-9 preparation plus the pyridine nucleotide regenerating system. Spectrophotometric grade dimethyl sulfoxide is obtained from Aldrich Chemicals.

Preparation of "S-9" Mix

S-9 mix contains per ml: $MgCl_2$ (8 μmoles), KCl (33 μmoles), NADP (4 μmoles), glucose-6-phosphate (5 μmoles), and sodium phosphate, pH 7.4 (100 μmoles). Stock solutions of NADP (0.1 M) and glucose-6-phosphate (0.1 M) are prepared with sterile water in sterile tubes and stored at -20°C. The stock salt solution (0.4 M $MgCl_2$, 1.65 M KCl) and phosphate buffer (0.125 M, pH 7.4) are autoclaved

Preparation of "S-9" Mix (Cont.)

and stored in the refrigerator. S-9 mix is prepared each day and is kept on ice before use. The amount of S-9 fraction added to this S-9 mix varies between 0.04 and 0.1 ml.

The optimal amount of S-9 added is determined by an experiment in which 10 µg α -benzopyrene is incubated (see preincubation section below) with increasing amounts of S-9 fraction (0.01 ml increments) in the presence of the S-9 mix components just described. Strain TA98 is used as the tester strain. After 20 minutes the preincubation mix is added to soft agar and poured over glucose agar plates (see spontaneous mutation section). After incubation and scoring the colonies, that concentration which gives the greatest number of revertants is used as the "optimal concentration" of S-9 fraction, which then is incorporated into the standard S-9 mix. This is usually about 50 µl. This is determined on each batch of liver homogenate initially, and after six months storage if any homogenate is left, when mutazyme preparation is used, 500 µl is incorporated into the preincubation mix.

Preincubation Procedure

Preincubation can detect mutagens such as dimethyl nitrosamine and pyrrolizidine alkaloids by putting mutagen, mutagen activating system and test organisms in close proximity to each other for a short time (3,4). Depending upon the solubility of the compound (soluble in water or in 50% DMSO), the compound is assayed after a preincubation period. The preincubation mix contained 0.1 ml of mutagen test solution, 0.5 ml of S-9 mix and 0.1 ml of bacterial culture, which are delivered, in the order given, to sterile glass tubes placed in a metal heating block. The tubes are incubated for 20 minutes

Preincubation Procedure (Cont.)

at 37°C and are shaken during the incubation.

Prescreening of Mutagen

Before a compound is assayed for mutagenicity, its solubility, toxicity, and possible mutagenicity is assessed.

The solubility of a compound is determined by placing 50 mg in a plastic screw-cap vial and adding 0.5 ml sterile distilled water with shaking. If the compound is not soluble in water, then dimethylsulfoxide is added dropwise up to 0.5 ml. Compounds soluble in water or in the 50% DMSO are preincubated with microsomes for assay (see preincubation procedure). If the compound is not soluble in water or 50% DMSO, its solubility in 100% DMSO is assessed by adding 0.5 ml DMSO to 50 mg of compound. If the compound is insoluble in DMSO, then its solubility is determined in ethylene glycol dimethyl ether, formamide, dimethylformamide, glycerol formal and acetonitrite. Compounds soluble in these solvents are added to soft agar so that the final concentration of solvent is not more than 200 µl/plate. Compounds not completely soluble in these solvents are assayed at the highest soluble level, which is determined.

After the solubility of the compound has been determined, a preliminary spot test is run on the compound to determine toxicity to growth and possible mutagenicity. This spot test is performed by adding 0.01 ml of concentrated compound in solvent to a small well inserted into soft agar containing S-9 mix and either strain TA98 or TA100 (both are used in duplicate). After 48 hours incubation, the plates are examined around the well for toxicity (no lawn growth) and for a ring of revertants.

Prescreening of Mutagen (Cont.)

We find the information from the spot test invaluable in determining the assay range of the compound. If the compound is not toxic and apparently not mutagenic, then higher levels (3, 5, 7, 10 mg/plate) will be used in a mutagen plate assay similar to that described in Table 1. For compounds which are toxic a "survival curve" is prepared in which varying levels of the compound are incorporated into glucose minimal agar (supplemented with a .05 mM biotin and histidine solution) and incubated with strains TA98 and TA100. Levels of compound which do not unduly inhibit growth are used in the mutagen plate assay similar to that described in Table 1 with the addition of plates to determine the viability of the test strain in the presence of each level at which the compound is tested.

α-Benzopyrene is utilized as the control mutagen for strains TA98, TA100, TA1537 and TA1538; 2-anthramine for strain TA1535. The use of these control mutagens allows the determination of the sensitivity of the standard strains. The efficacy of the activation system and preincubation is tested by the use of dimethyl nitrosamine with strain TA100. Each assay requires 10 conditions, 2 activation conditions (with and without rat liver homogenate) and 3 strains in duplicate.

Mutagen Handling

Mutagens are stored on a spill tray in a hood used only for this purpose. Mutagens are handled in disposable plastic test tubes using the aid of propipets with disposable tips. The Bioassay Room contains a ventilating hood under which all manipulations with mutagens are performed. Disposable plastic gloves are used in all manipulations involving mutagens which are handled in a spill tray. All waste contaminated by mutagens as well as mutagens are stored in a sealed drum located near the hood and are removed to a Class I disposal site. We also have a ventilating fan attached to the incubators which can remove the incubator air before the incubator door is opened.

Standard Assay Protocol

The standard assay protocol is the procedure by which candidates are screened for mutagenicity (Table 2). It incorporates all the procedures previously described. In essence, upon receipt of a compound it is stored in the mutagen hood and prescreened (see prescreen section, p.8). Upon determination of dose range, an assay is initiated using tester strains whose phenotype has been determined to be suitable (see phenotype section, p. 2). Manipulations similar to those described in the Spontaneous Mutation Frequency Section(p.4) and the induced Mutation Section (p. 5) are used. For compounds which are soluble in water or DMSO, a preincubation (see p. 7) is used before adding the mixture to top agar. Each assay is manipulated as described in Table 2 and contains baseline controls, 4 levels of unknown mutagen and known mutagen controls.

TABLE 1

	Condition	Culture Dilution	Expected Number Of Bacteria Per Plate	Top Agar Supplemented
1	Pre-existing mutants	10^{-1}	10^{7}	0.05 mM biot
2	Viable count	10^{-6}	10^{2}	0.05 mM biot 3.14 mM his
3	Spontaneous plate mutants	10^{-1}	10^{7}	0.05 mM biot 0.05 mM his
4	Control without solvent	10^{0}	10^{8}	0.05 mM biot 0.05 mM his
5	Solvent control	10^{0}	10^{8}	0.05 mM biot 0.05 mM his

TABLE 2

	Condition	Culture Dilution	Expected Number Of Bacteria Per Plate	Top Agar Supplemented
A)	Viable Count	10^{-6}	10^{2}	0.05 mM biot 3.14 mM his
B)	Spontaneous plate mutants	10^{-1}	10^{7}	0.05 mM biot 0.05 mM his
C)	Mutagen 1 + S-9	10^{0}	10^{8}	0.05 mM biot 0.05 mM his
D)	Mutagen 2 + S-9	10^{0}	10^{8}	0.05 mM biot 0.05 mM his
E)	Mutagen 3 + S-9	10^{0}	10^{8}	0.05 mM biot 0.05 mM his
F)	Mutagen X Level 1 + S-9	10^{0}	10^{8}	0.05 mM biot 0.05 mM his
G)	Mutagen X Level 2 + S-9	10^{0}	10^{8}	0.05 mM biot 0.05 mM his
H)	Mutagen X Level 3 + S-9	10^{0}	10^{8}	0.05 mM biot 0.05 mM his
I)	Mutagen X Level 4 + S-9	10^{0}	10^{8}	0.05 mM biot 0.05 mM his
J)	Mutagen X Level 4	10^{0}	10^{8}	0.05 mM biot 0.05 mM his

TABLE 3

STANDARD ASSAY PROTOCOL

(preincubated control mutagen - 3 strains in duplicate)

Agar Overlay Composition

Plate	100 µl TA98	100 µl TA100	100 µl TA1537	500 µl S-9 Mix	200 µl a	100 µl b	100 µl c	200 µl d	Condition
1	+							+	A
2		+						+	A
3			+					+	A
4	+				+				B
5		+			+				B
6			+	+	+	+			B
7	+			+	+	+			C
8		+		+	+	+			C
9		+		+	+		+		D
10		+		+	+			+	E

a) .05 mM histidine - .05 mM biotin
b) α-Benzopyrene
c) Dimethyl nitrosamine
d) .05 mM biotin - 3.14 mM histidine

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TABLE 3 (Cont.)

STANDARD ASSAY PROTOCOL

(preincubated mutagen - 4 levels - 3 strains in duplicate)

Agar Overlay Composition

Plate	100 µl TA98	100 µl TA100	100 µl TA1537	200 µl .05 mM H .05 mM B	100 µl UK [1]	100 µl UK [2]	100 µl UK [2]	100 µl UK [3]	500 µl S-9 Mix	Condition
11	+			+	+				+	F
12		+		+	+				+	F
13			+	+	+				+	F
14	+			+		+			+	G
15		+		+		+			+	G
16			+	+		+			+	G
17	+			+			+		+	H
18		+		+			+		+	H
19			+	+			+		+	H
20	+			+				+	+	I
21		+		+				+	+	I
22			+	+				+	+	I
23	+			+				+		J
24		+		+				+		J
25			+	+				+		J

LITERATURE CITED

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4. Prival, M., A. Shelton and V. King. 1979. 10th Ann. Mtg Environ. Mut. Soc. New Orleans, pp 67-68.
5. Seiler, J. P., I. E. Mattern, M. H. L. Green and D. Anderson. 1980. Mutation Research 74:71-75.
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QUALITY ASSURANCE CHECKLIST

FOR THE MUTAGEN TEST ASSAY I & II

Source:

Material:

Laboratory No:
Date Received:
Date Completed:
Study Director:
Technician:
QA Monitor:

Appearance of inoculum on master plates:

TA98: ______________________________

TA100: ______________________________

TA1535: ______________________________

TA1537: ______________________________

TA1538: ______________________________

Prescreen of mutagen: ____________________

Assay:

Date Started: ______________________

Date Counted: ______________________

Results:

Strain	Condition	Mutagen	Revertants/plate

Comments: